Behavioural profiles and cellular mechanisms of retinoid-induced depression

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Table of contents

Table of figures and tables

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Abstract

Vitamin A and its derivatives, known as retinoids, are involved in a number of functions in the developing and adult brain (Lane *et al.*, 2005). Roaccutane (13-*cis*-retinoic acid, 13-*cis*-RA) is a synthetic retinoid used for the treatment of severe cystic acne, although its use has been controversially associated with adverse psychiatric events including depression. In this thesis, the presence of retinoid receptors in the rat hippocampus was verified and a similar profile of expression was observed in the rat raphe nuclei for the first time. The expression of retinoid receptors in brain regions that are implicitly associated with depression pathology provides proof of concept for retinoids to influence depressive behaviour.

The ability of 13-*cis*-RA treatment to induce a pro-depressive profile in animal models of depression-related behaviour was tested. In the resident-intruder paradigm, adult rats treated for 7 or 14 days with 13-*cis*-RA (1mg/kg, i.p.) showed reduced aggressive behaviour, with a concomitant increase in flight submit and flight escape behaviours, compared with vehicle-treated controls. These findings are indicative of increased depression-related behaviour. However, chronic treatment did not alter depression-related behaviour in the forced swim test and sucrose consumption anhedonia paradigms.

The molecular mechanisms mediating 13-*cis*-RA-induced depression were investigated by examining monoaminergic gene expression, protein levels and neurotransmitter levels in rat brain tissue and plasma and an *in vitro* model. The majority of serotonergic components (SERT, 5-HT_{1A}R, 5- HT_{1B}R and MAOA) were not altered by chronic 13-*cis*-RA treatment, with the possible exception of TPH2 gene/protein expression and increased 5-HT levels in platelets. In fact, the expression of D2 dopamine receptor was significantly elevated in the RN46A-B14 cell line (10µM 13-*cis*-RA, 48 h) and was similarly elevated at the protein level in the juvenile rat hippocampus (1mg/kg/day, i.p., 6 weeks), suggesting dopaminergic pathways may be of importance. There was also a trend in the data to suggest that 13-*cis*-RA-treated juvenile rats may be more susceptible the molecular alterations than corresponding adult rats.

Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
5-HTT	5-HT reuptake transporter
5-HTP	5-Hydoxytryptophan
8-OH-DPAT	8-hydroxy-2(di-n-propylamino)tetralin
13-*CIS*-RA	13-*cis*-retinoic acid
ADERS	Adverse drug event reporting systems
APOE	Apolipoprotein E
APP	Amyloid plaque precursor
ATRA	All-*Trans*-Retinoic Acid
BCA	Bicinchinic acid
BDNF	Brain derived neurotrophic factor
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CLN	Caudal linear nucleus
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
CRABP	Cellular Retinoic Acid Binding Protein
CRABP-I	Cellular Retinoic Acid Binding Protein I
CRABP-II	Cellular Retinoic Acid Binding Protein II
CRBP-I	Cellular Retinol-Binding Protein Type I
CRBP-II	Cellular Retinol-Binding Protein Type II
CRH	Corticotropin-releasing hormone
CSF	Cerebrospinal fluid
D1DR	D1 dopamine receptor
D2DR	D2 dopamine receptor
D3DR	D3 dopamine receptor
D4DR	D4 dopamine receptor
D5DR	D5 dopamine receptor
DA	Dopamine
DMEM	Dulbecco Modified Eagles's minimum essential medium
DMSO	Dimethyl sulphoxide
DOPA	Dihydroxyphenylalanine
DOPAC	4-Dihydroxyphenylacetic acid
DR	Direct Repeats
DRN	Dorsal raphe nucleus
FBS	Foetal bovine serum
FDA	Food and drug administration
FST	Forced Swim Test
GABA	γ-aminobutyric acid
GLUR	Glutamate receptor
GOI	Gene of interest
GPCR	G protein coupled receptor
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase Complex
HIPP	Hippocampus

HPLC	High-performance liquid chromatography
HPA	Hypothalamic-pituitary-adrenal
HVA	Homovanillic acid
IU	International units
L-DOPA	L-Dihydroxyphenylalanine
LRAT	Lecithin:Retinol Acyl Transferase
MAO	Monoamine oxidase
MAOA	Monoamine oxidase A
MAOB	Monoamine oxidase B
MAOI	Monoamine oxidase inhibitor
MRN	Median raphe nucleus
nBLAST	nucleotide Basic Local Alignment Search Tool
N-CoR	Nuclear Receptor Corepressor
NA	Noradrenaline
NaCl	Sodium Chloride
NR	NMDA receptor
PET	Positron emission tomography
PFCX	Prefrontal cortex
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RALDH	Retinal Dehydrogenase
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Response Elements
RBP	Retinol Binding Protein
RIPA	Radio Immuno Precipitation Assay
ROLDH	Retinol Dehydrogenase
RXR	Retinoid X Receptors
SDS-PAGE	Sodium dodecyl sulphate-polyacylamide gel electrophonesis
SERT	Serotonin re-uptake transporter
SNP	Single-nucleotide polymorphism
SR-B1	Scavenger receptor class B type
SRC	Steroid Receptor Coactivator
SSRI	Selective serotonin re-uptake inhibitor
TBST	Tween tris buffered saline
TCA	Tricyclic antidepressant
TEMED	Tetramethylethylenediamine
TPH	Tryptophan hydroxylase
TPH2	Tryptophan hydroxylase 2
TST	Tail Suspension Test
TTR	Transporter Transthyretin
VAD	Vitamin A deficiency
VMAT	Vesicular monoamine transporter
VNTR	Variable-number-of-tandem-repeat
VTA	Ventral tegmental area

Chapter 1

General Introduction

1.1. Introduction

Retinoids are vitamin A derived compounds that mediate a number of physiological functions including well established roles in central nervous system (CNS) development, such as the patterning of the anterior-posterior and dorsoventral axes (Maden, 2002). However, accumulating evidence suggests the adult brain is also receptive to retinoids through a network of retinoid specific receptors and signalling pathways (Lane *et al.*, 2005; Mey *et al.*, 2004). This emerging field of research has suggested that a multitude of functions including memory and learning (Cocco *et al.*, 2002), synaptic plasticity (Chiang *et al.*, 1998; Misner *et al.*, 2001), sleep (Tafti *et al.*, 2007) and neurological diseases (Lane *et al.*, 2005) are influenced by retinoids. The research in our laboratory is primarily concerned with the ability of retinoids to alter mood and emotional responsiveness (O'Reilly *et al.*, 2008) with particular focus on the synthetic retinoid 13-*cis*-retinoic acid (13-*cis*-RA), marketed as Roaccutane and Accutane for the treatment of severe cystic acne.

Since the introduction of 13-*cis*-RA onto the market in 1982, approximately 5-10% of 13-*cis*-RA patients have experienced cases of depression, suicide ideation and completed suicide (Bremner *et al.*, 2007; Hull *et al.*, 2005). In support of the pro-depressive properties of 13-*cis*-RA, a number of reports have noted that excessive retinoid intake, known as hypervitaminosis A, can lead to incidences of adverse psychiatric events (McCance-Katz *et al.*, 1992; Rodahl *et al.*, 1943). However, the ability to definitively establish 13-*cis*-RA treatment as a causal factor of adverse psychiatric events has remained elusive and controversial (Strahan *et al.*, 2006), particularly as the largest population study to date has found no such association (Jick *et al.*, 2000).

Previous work in our laboratory has shown that juvenile male DBA/2J mice, treated daily with 13-*cis*-RA for 6 weeks, exhibit increased depressive indices in two animal model of depression-related behaviour called the forced swim test (FST) and tail suspension test (TST) (O'Reilly *et al.*, 2006). However, other studies in adult rats have shown that 13-*cis*-RA treatment for 6 weeks has no effect in depression-related behaviour as measured by the FST and sucrose consumption test (Ferguson *et al.*,

2005a; Ferguson *et al.*, 2007b) . This may be largely attributable to a difference in methodology between the two studies, particularly drug dose and route of administration, or may indicate species or age related sensitivity to the effects of 13-*cis*-RA.

Retinoids have well documented roles in regulating gene transcription (McGrane, 2007). Of particular interest is the ability of retinoids to regulate neuronal gene transcription and this has led to the suggestion that 13-*cis*-RA treatment may exert cellular effects that underlie changes in behaviour. Further information on retinoids, how they signal in the brain and mediate gene transcription, and their potential role in depression is discussed below.

1.2. Vitamin A and retinoids

Vitamin A is defined as any compound with the biological activity of all-*trans*-retinol (IUPAC-IUB, 1982) (Figure 1.1A), a primary alcohol compound consisting of a cyclohexenyl ring with a side chain containing 4 *trans* double bonds and an alcohol end group. Humans, along with all animal species, are incapable of *de novo* vitamin A synthesis (Goodman, 1984) and must therefore obtain ~5000 international units (IU) per day of dietary vitamin A (Food and Nutrition Board of the National Research Council, 1980). Foods rich in vitamin A include plant-derived foods such as yellow, orange and red vegetables and fruits (in the form of carotenoids) and animal-derived foods such as liver, butter and milk (in the form of retinyl esters) (Blomhoff *et al.*, 2006). Vitamin A has been found to be an essential requirement in the visual process (Wald, 1968), maintenance of epithelial surfaces (Wolbach *et al.*, 1978) and many other physiological functions (reviewed in (Blomhoff *et al.*, 2006)).

The importance of this micronutrient is further highlighted by the 3 million people worldwide who suffer from vitamin A deficiency (VAD), many of whom are children from developing countries (World Health Organisation, 1995). The symptoms of vitamin A deficiency include ocular impairments such as night blindness, xerophthalmia (dry eyes), as well as generally increased morbidity and mortality rates through increased incidence of respiratory disease (Bendich *et al.*,

1989). Work to prevent vitamin A deficiency has come mainly in the form a genetically modified variety of *Oryza sativa* rice, known as golden rice, that biosynthesizes a precursor of pro-vitamin A in the edible parts of rice known as the endosperm (Ye *et al.*, 2000).

The term 'retinoids' has been in use since 1976 (Sporn *et al.*, 1976) and has been applied to structural derivatives of retinol that arise through modifications at the functional terminal group. For instance, all-*trans*-retinol (Figure 1.1A) is converted to retinal (also known as retinaldehyde, Figure 1.1B) through oxidation of the alcohol end group, while further oxidation of the aldehyde end group of retinal creates all-*trans*-retinoic acid (ATRA, Figure 1.1C), a highly important bioactive retinoid. The term retinoids encompasses these retinol analogues (irrespective of biological activity) in addition to compounds that are structurally unrelated to retinol but elicit biological vitamin A (or retinoid) activity. In addition, retinoids can be divided as either naturally occurring forms obtained from foodstuffs or synthetic forms that are utilised as drug compounds in the field of dermatology, cancer and metabolic disease (Altucci *et al.*, 2007). The following sections describe the synthesis, transport and signalling of natural retinoids followed by their functions in the adult brain.

1.2.1. Vitamin A intake, metabolism and transport

Plants, bacteria and algae synthesize around 400 isoprenoid pigments known as carotenoids (Fraser *et al.*, 2004) and a smaller group of these such as β-carotene (Figure 1.1E), α-carotene, and β-cryptoxanthin have pro-vitamin A activity. This signifies that upon ingestion and absorption into the enterocytes of the small intestine, carotenoids are converted into either retinal or are further reduced to retinol. Similarly, retinyl esters (Figure 1.1D) derived from ingestion of animal tissue are converted into retinol and therefore makes retinol the main storage and transport source of vitamin A in the body.

Ingested carotenoids are known to be absorbed into enterocytes of the small intestine lumen by the lipid transporter scavenger receptor class B type I (SR-B1) (During *et al.*, 2007). β-Carotene then

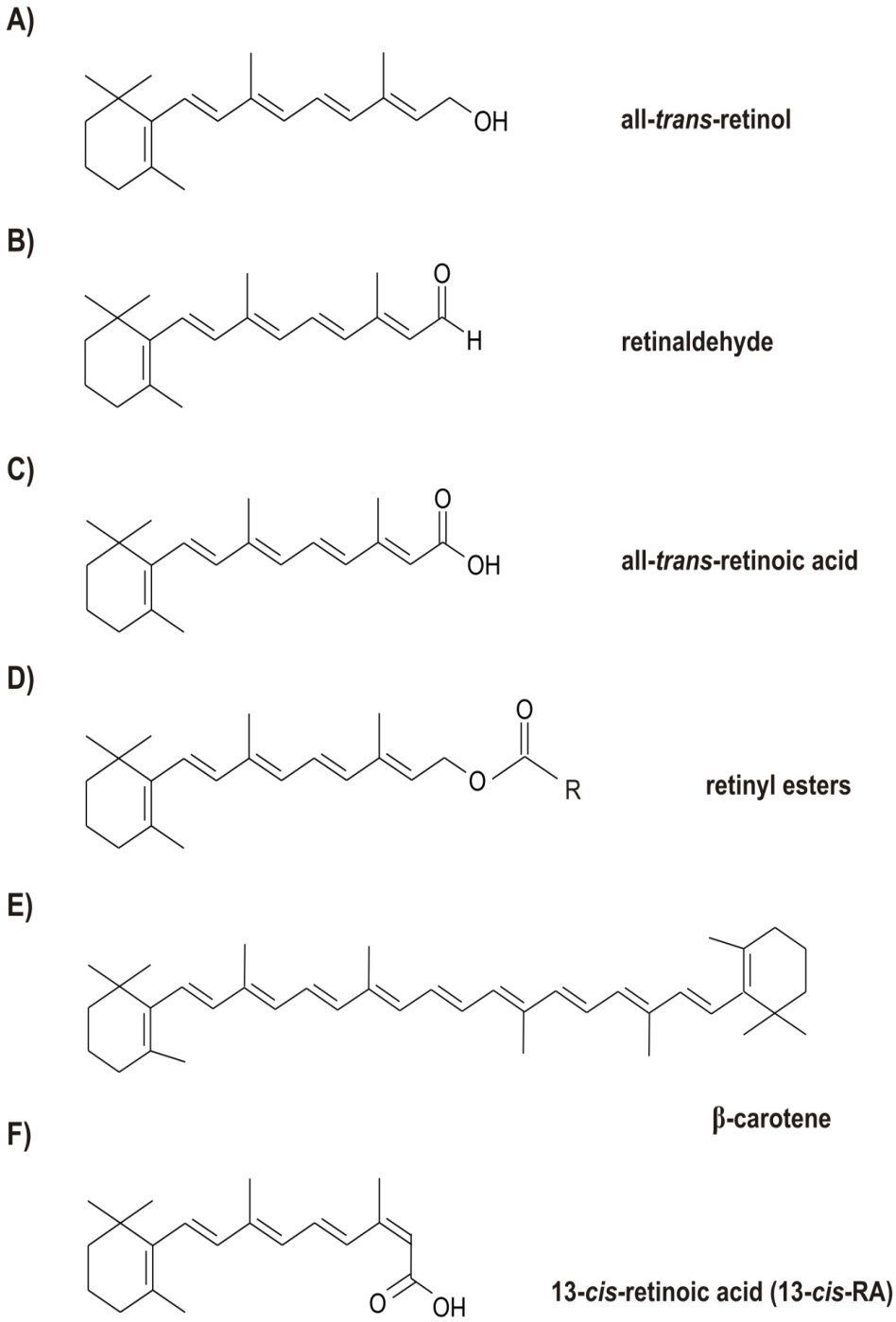

Figure 1.1: Structure of retinoid compounds. Ingested all-*trans*-retinol (A) is converted into retinaldehyde (B) by retinol dehydrogenase and subsequently converted to all-*trans*-retinoic acid (C) by retinal dehydrogenase. Stored all-*trans*-retinol can also take the form of retinyl esters (D), while the carotenoid β-carotene (E) has the highest pro-vitamin A activity of all carotenoids and is common in plant-based foodstuffs. Closely related to all-*trans*-retinoic acid, 13-*cis*-retinoic acid (F) is a synthetic retinoid. Diagram constructed using MDL ISIS/Draw 2.5 and redrawn from Bremner *et al.*, 2007.

appears to be converted to retinal by symmetric/asymmetric cleavage (Goodman *et al.*, 1965; Napoli *et al.*, 1988; Olson *et al.*, 1965). Retinal is reduced to all-*trans*-retinol in the enterocytes by a retinal dehydrogenase, although it has yet to be fully identified *in vivo* (Li *et al.*, 2003). Meanwhile, when animal tissues are ingested, retinyl esters are hydrolysed to retinol by pancreatic triglyceride lipase in the lumen and phospholipase B in the brush membrane border (Rigtrup *et al.*, 1994) and pass into the enterocyte by passive diffusion (During *et al.*, 2007).

In the enterocytes, retinol is bound to cellular retinol-binding protein type II (CRBP-II) (Crow *et al.*, 1985). Once bound, CRBP-II facilitates the esterification of retinol with fatty acids such as palmitate by the enzyme lecithin:retinol acyltransferase (LRAT) (Herr *et al.*, 1992). The majority of these retinyl esters are subsequently incorporated into aggregates of triacylglycerol and phospholipids along with carotenoids, retinol and specific apolipoproteins, collectively known as nascent chylomicrons (Blomhoff *et al.*, 1982). The nascent chylomicrons then secrete retinyl esters, mainly in the form of retinyl palmitate, into the lymphatics, although a significant amount of unesterified all-*trans*-retinol is secreted into the portal circulation (Harrison, 2005).

During the circulation of nascent chylomicrons through the lymph and blood, triacylglycerol hydrolysis occurs through lipoprotein lipase and the apolipoprotein cofactor (apo)C-I1, as well as the addition of apolipoprotein E (apoE) to the now mature chylomicrons. The addition of apoE to the chylomicron signals its removal by the liver, thereby creating chylomicron remnants (reviewed in (Cooper, 1997)). The chylomicron remnants, still containing retinyl esters, are subsequently cleared by the hepatocytes of the liver (Blomhoff *et al.*, 1982), although other extra-hepatic locations such as bone marrow may also process chylomicron remnants (Paik *et al.*, 2004).

In the hepatocytes, the retinyl esters are either hydrolysed to all-*trans*-retinol where it is associated with the lipocalin retinol binding protein (RBP) (Kanai *et al.*, 1968) or transferred to perisinusoidal stellate cells for storage (Blomhoff *et al.*, 1984). The all-*trans*-retinol-RBP complex is translocated from the hepatocyte endoplasmic reticulum (Ong *et al.*, 2000) to the golgi complex before being

released into the plasma, where it associates with the plasma transporter transthyretin (TTR) in a 1:1 ratio (Peterson, 1971). In addition to all-*trans*-retinol, other retinoid metabolites have been reported to be present in plasma such as all-*trans*-retinoic acid, 13-*cis*-4-oxo retinoic acid and all-*trans*-4-oxo retinoic acid (Wyss *et al.*, 1997). Upon reaching the target cell, intracellular uptake of all-*trans*-retinol occurs through the association of RBP with the STRA6 membrane receptor (Kawaguchi *et al.*, 2007).

1.2.2. Active retinoids

1.2.2.1. Intracellular retinoid synthesis

All-*trans*-retinol, synthesized from ingested precursors, is transported and stored in target cells when required (shown in Figure 1.2). All-*trans*-retinol bound to RBP is lipophilic and is therefore able to pass through the plasma membrane of the target cell. The uptake of lipoproteins (containing retinyl esters, retinol and carotenoids) or ATRA and its metabolites from plasma may occur via additional pathways into the cell (Blomhoff *et al.*, 2006). Cellular retinol binding protein I (CRBP-I) associates with all-*trans*-retinol and facilitates the uptake of all-*trans*-retinol into the target cell (Vogel *et al.*, 2001). All-*trans*-retinol is subsequently converted to retinyl esters by LRAT for storage or converted into ATRA (reviewed in (Napoli, 1996)).

ATRA is the most prominent cellular retinoid, mediating the majority of the biological effects of Vitamin A. It is derived from all-*trans*-retinol via a two step oxidation process. The first reaction is catalysed by retinol dehydrogenases (ROLDHs) such as alcohol dehydrogenases 1,2 and 4 and short-chain dehydrogenase/reductases (Duester *et al.*, 2003) and convert all-*trans*-retinol to retinal. Secondly, retinal is further oxidised to ATRA by retinal dehydrogenases (RALDHs), particularly in the form of RALDH2 found in many cell types (Niederreither *et al.*, 1997). Newly synthesised ATRA associates with cellular retinoic acid binding proteins (CRABPs) CRABP-I and CRABP-II (Dong *et al.*, 1999) that enable the intracellular transport of ATRA to other subcellular locations or into the cell nucleus, where they mediate their functional effects (see Figure 1.2).

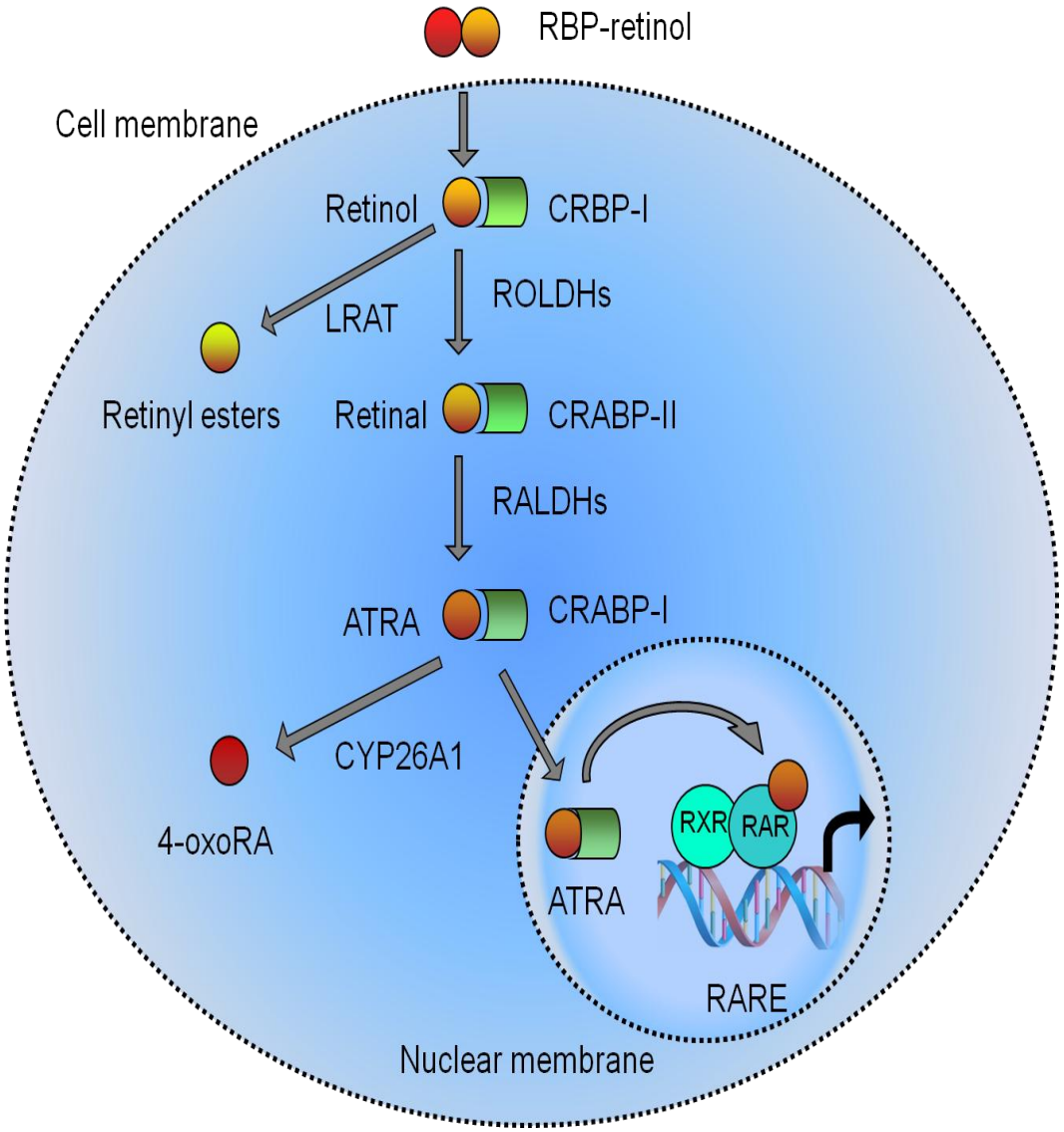

Figure 1.2: Intracellular retinoid signalling. Retinol binding protein (RBP) transports all-*trans*-retinol to the target cell. All-*trans*-retinol is subsequently transported intracellularly by cellular retinol binding proteins (CRBP-I). All-*trans*-retinol can then be converted to retinyl esters for storage by the enzyme lecithin:retinol acyltransferase (LRAT) or oxidised to retinal/retinaldehyde by retinol dehydrogenases (ROLDHs). Retinal bound to cellular retinoic acid binding protein II (CRABP-II) is further oxidised by retinal dehydrogenases (RALDH) to form all-*trans*-retinoic acid (ATRA). ATRA bound to cellular retinoic acid binding protein I (CRABP-I) passes through the nuclear membrane, whereby ATRA can bind to retinoic acid receptors (RARs) that form heterodimers with retinoid X receptors (RXRs). Together they bind to retinoic acid response elements (RAREs) and activate gene transcription. Other pathways for ATRA exist, such as the metabolism to all-*trans*-4-oxoretinol by the enzyme CYP26A1 (Lane *et al.*, 1999). Adapted from Lane *et al*., 2005.

The adult brain is thought to be capable of active retinoid synthesis (Lane *et al.*, 2005; McCaffery *et al.*, 2006; Mey *et al.*, 2004). RBP bound with all-*trans*-retinol is able to traverse the blood brain barrier (MacDonald *et al.*, 1990) and ATRA synthesis has been shown in the adult brain of the rat (Werner *et al.*, 2002), mouse (Wagner *et al.*, 2002) and rabbit (Dev *et al.*, 1993). Indeed, the synthesis of ATRA in the cerebrum, cerebellum and meninges of adult rabbits was equivalent or exceeded ATRA synthesis in the rat liver (Dev *et al.*, 1993). Further studies have shown the distribution of CRBP-I and CRABP-I in the olfactory bulb, caudate, nucleus accumbens, hippocampus, amygdala, cortex and hypothalamus, while CRABP-II distribution is restricted to the nucleus caudate, accumbens and septum (Zetterstrom *et al.*, 1999).

1.2.2.2. Nuclear retinoid receptors

The differential binding of ATRA to either CRABP-I or II appears to create divergent responses. ATRA bound to CRABP-I decreases cellular responses to ATRA by catalysing ATRA degradation (Fiorella *et al.*, 1993). However ATRA bound to CRABP-II initiates translocation into the cell nucleus allowing ATRA to bind to retinoic acid receptors (RARs) (Delva *et al.*, 1999). The CRABP-II -RAR complex mediates ligand ‘‘channeling’’ that facilitates the ligation of ATRA to the RAR (Budhu *et al.*, 2002). RARs belong to the steroid/thyroid hormone receptor family and heterodimerize with retinoid X receptors (RXRs). The RARα, β and γ subtypes have been identified and additional receptor isoforms of each subtype were found to exist due to gene splicing (RARα1, α2, β1, β2, β3, β4, γ1 and γ2) (reviewed in (Chambon, 1996)). RXR α, β and γ are closely related nuclear receptors that also exist as different isoforms (RXRα1, α2, β1, β2, γ1 and γ2) (Mangelsdorf *et al.*, 1990).

Both RARs and RXRs have a distinct pattern of distribution in the cell nuclei of neuronal cells within the adult brain (Krezel *et al.*, 1999), as highlighted by *in situ* hybridization and immunolabelling studies in adult mice (shown in Figure 1.3. and Figure 1.4., respectively). RARα has particularly high expression within the cortex (cingulate, frontal and parietal) and hippocampus (fields CA1, 2 and 3) and is also expressed within the olfactory bulb, amygdaloid basolateral and lateral nuclei, thalamus,

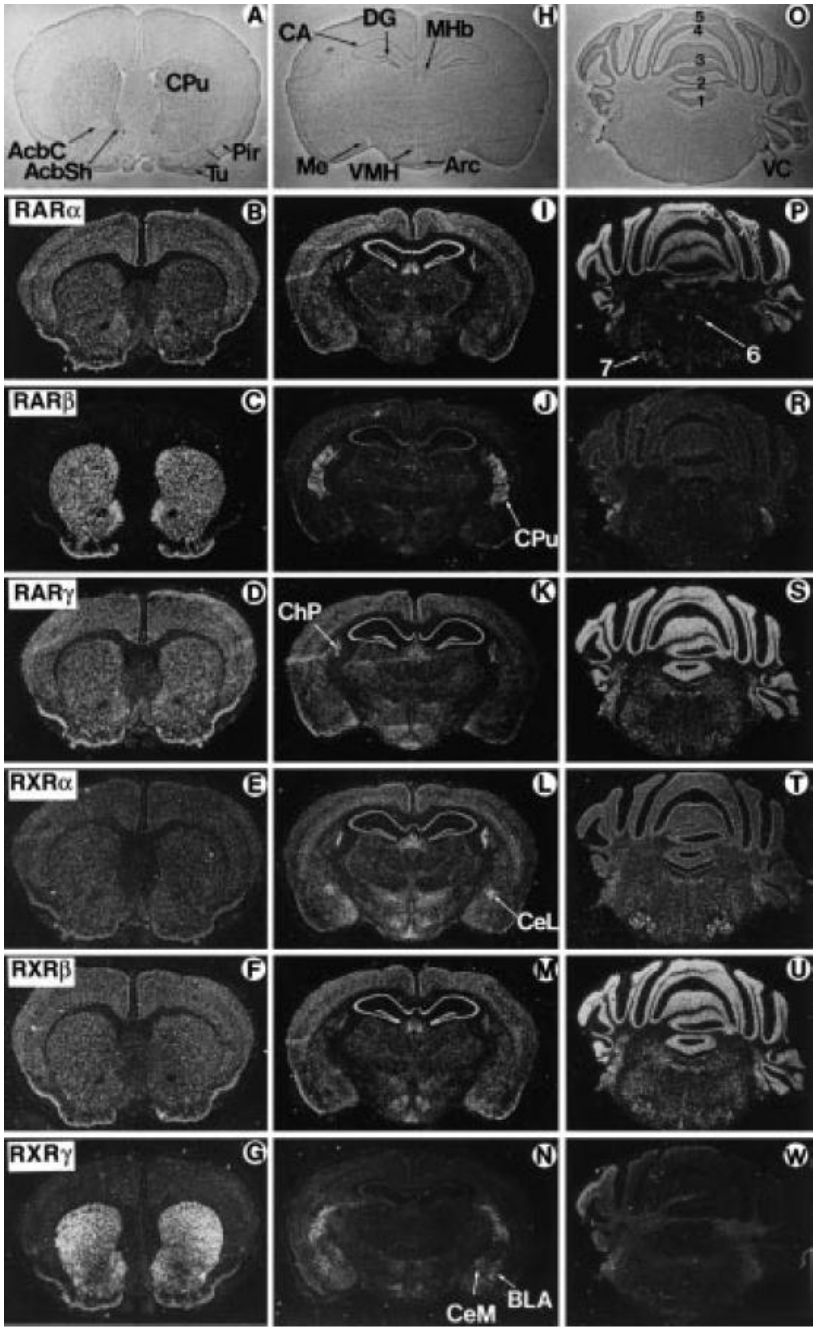

Figure 1.3: The expression of retinoid receptor mRNA transcripts in the adult mouse brain. Coronal sections through the caudate–putamen and nucleus accumbens (A), hippocampus and arcuate hypothalamus (H) and cerebellar lobules, abducens and facial nuclei (O) are presented in the top row as bright-field views. The corresponding dark-field *in situ* hybridization views are shown for RARα (B, I, P), RARβ (C, J, R), RARγ (D, K, S), RXRα (E, L, T), RXRβ (F, M, U) and RXRγ (G, N, W). Arc = arcuate hypothalamus, AcbC = nucleus accumbens core, AcbSh = nucleus accumbens shell, BLA = basolateral amygdaloid nucleus, anterior, CA = fields CA1–3 of Ammon's horn, CeL = central amygdaloid nucleus, CeM = central amygdaloid nucleus, medial division, ChP = choroid plexus, CPu = caudate putamen, DG = dentate gyrus, Me = medial amygdaloid nucleus, MHb = medial habenular nucleus, Pir = piriform cortex, Tu = olfactory tubercle, VC = ventral cochlear nucleus, VMH = ventromedial hypothalamic nucleus, 1-6 = cortical layers and 7= facial nucleus. Taken from Krezel *et al*., 1999.

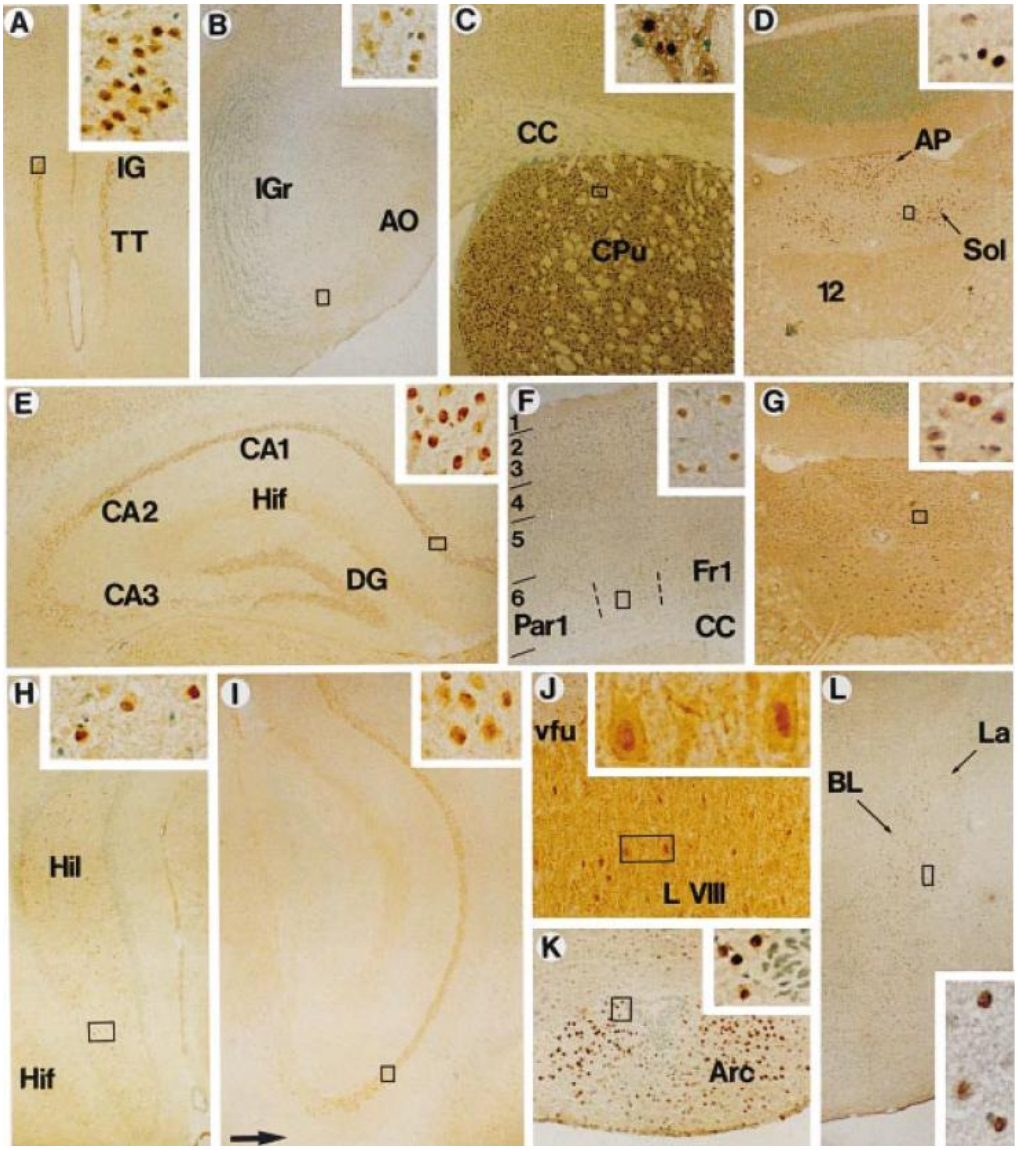

Figure 1.4: The expression of retinoid receptor proteins in the adult mouse brain. The immunohistochemical detection of RARα (A, B), RARβ (C, D), RARγ (E, F), RXRα (G, H), RXRβ (I, J) and RXRγ (K, L) was conducted in coronal sections through the tenia tecta (A), main olfactory bulb (B), caudate–putamen (C), solitary tract and hypoglossal nucleus (D, G), left and right hippocampus (E, I), neocortex (F), hilus of the dentate gyrus (H), cervical spinal cord (J), arcuate hypothalamus (K) and basolateral amygdaloid nucleus (L). Magnification was x40 in all cases except J and K, with a x100 magnification. The staining in selected regions, represented by a square, is shown at x400 magnification in a corner of each panel. The dorsal region is at the top of all panels, except in panel I where it is on the right, indicated by the arrow. Descriptions of panels G and I are equivalent to those in panel D and E, respectively. AO = anterior olfactory nucleus, AP = area postrema, Arc = arcuate hypothalamus, BL = basolateral amygdaloid nucleus, CA fields CA1–3 of Ammon's horn, CC = corpus callosum, CPu = caudate putamen, DG = dentate gyrus, FR1 = frontal cortex, area 1, Hif = hippocampal fissure, Hil = hilus of the dentate gyrus, IG = indusium griseum, IGr = internal granular layer of the olfactory bulb, La = lateral amygdaloid nucleus, L VIII = lamina VIII of the spinal cord, Par1 = parietal cortex, Sol = solitary tract nucleus, TT = tenia tecta, vfu = ventral funiculus of spinal cord, 1-6 = cortical layers and 12 = hypoglossal nucleus. Taken from Krezel *et al*., 1999.

pons, pituitary and many other structures (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). RARβ has a restricted distribution within the caudate/putamen, nucleus accumbens and dorsomedial hypothalamic nucleus, while RARγ has very low expression in the diencephalic and rhombencephalic regions, with the notable exception of the hippocampus.

Meanwhile, RXRα has been detected in the hippocampus, medulla oblongata, pons, pituitary and numerous cortical regions, all at low levels (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). In contrast, the distribution of RXRβ is limited to the cingulate cortex, hippocampus, striatum, thalamus and pituitary, while RXRγ is found in the striatum, caudate-putamen, shell and core of the nucleus accumbens and hypothalamus (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). It is therefore apparent that the hippocampus, a brain region extensively explored in this thesis, contains most RAR and RXR subtypes, in addition to CRBP I and CRAPB I (Zetterstrom *et al.*, 1999) that is suggestive of a fully functioning retinoid signalling system in this region. Less is known about the expression of these retinoid signalling components in the raphe nuclei, the other brain region thoroughly examined in this thesis, although CRABP I mRNA expression was detected (Zetterstrom *et al.*, 1999), which may indicate the presence of additional retinoid signalling components in this region.

The ligand specificities of RARs and RXRs differ considerably. RARs signal through the high affinity ligand binding of ATRA (Soprano *et al.*, 2004) and upon binding, a small population of RARs may translocate from cytoplasmic locations to the nucleus (Maruvada *et al.*, 2003). The specific ligand for RXRs was reported to be 9-*cis*-RA *in vitro* (Zhang *et al.*, 1992b), although 9-*cis*-RA has not been detected in any tissues *in vivo* and may therefore be physiologically irrelevant (Mic *et al.*, 2003). Additionally, RXRs do not necessarily require ligand binding for activation (Rowe, 1997), acting as cofactors for thyroid hormone receptors (Bugge *et al.*, 1992) and the vitamin D receptors (Kliewer *et al.*, 1992). Importantly, RXRs serve as heterodimeric partners for RARs (Zhang *et al.*, 1992a). ATRA binding to the RAR causes conformation changes within the ligand-binding domain that favours RAR-RXR heterodimerization (Rochette-Egly *et al.*, 2009) in a process known as 'RAR dominance' (Kurokawa *et al.*, 1994). Given that the RAR-RXR heterodimer is then able to regulate gene

transcription, RARs can be viewed as ligand-dependent transcription factors that mediate the effects of ATRA.

13-*Cis*-RA, the active ingredient in Roaccutane, is thought to mediate its cellular effects via the same signalling pathways as the endogenous retinoid ATRA. 13-*Cis*-RA has been shown to bind directly to RARα, RARβ and RARγ, although with a low binding efficiency when compared with ATRA (micromolar range compared with nanomolar range, respectively) and does not bind to RXR at all (Idres *et al.*, 2002). Furthermore, 13-*cis*-RA is known to be rapidly isomerised to ATRA in cultured SZ95 sebocytes over the course of 6 h (Tsukada *et al.*, 2000) and may therefore exert its effects as ATRA itself, by binding to RARs (as described previously).

1.2.2.3 Retinoid-induced gene transcription

RARs and RXRs are characterised by *i)* a variable NH2-terminal region with ligand-independent activation function, *ii)* a conserved DNA binding domain that allows for DNA recognition and binding, *iii)* a hinge region and *iv)* a multi-functional C-terminal ligand-binding domain with ligand-dependent activation function (Glass *et al.*, 2000). The DNA-binding domain contains two zinc-binding motifs and two α helices that fold in a globular conformation (Lee *et al.*, 1993) and confers sequence-specific DNA binding to retinoic acid response elements (RAREs). RAREs are present in the promoters of target genes (de The *et al.*, 1990) and usually consist of direct repeats (DR) of the consensus half-site motif AGGTCA (or TGACC), spaced by either 1, 2 or 5 base pairs (DR1, DR2 and DR5, respectively). DR2 and DR5 elements preferentially bind RXR-RAR heterodimers with the RXR binding to the 5' half-site and RAR to the 3' half site, while DR1 bind with the reverse polarity (Rastinejad *et al.*, 2000).

Ligand binding causes structural changes in the ligand-dependent activation function of RARs (Renaud *et al.*, 1995) that in turn leads to the release of corepressors including the nuclear receptor

corepressor and the silencing mediator for retinoid and thyroid hormone receptors (Chen *et al.*, 1995) and the recruitment of steroid receptor coactivators (SRC-1,2 and 3) (Darimont *et al.*, 1998). Both coactivators and corepressors recruit proteins that moderate the acetylation of histones surrounding the DNA through the RARE: histone acetyl transferase complex and RARE:histone deacetylase complex, respectively. Coactivators decompact histones through the acetylation of lysine residues, followed by the recruitment of transcriptional machinery via the association of RAR-RXR with the *S*rb and *M*ediator protein *c*ontaining *c*omplex (Dilworth *et al.*, 2001). This mediator complex facilitates the entry of RNA polymerase II to the promoter transcription start site at the TATA box, thus beginning transcription of the target gene (Woychik *et al.*, 2002).

More than 500 genes have been suggested to be regulated by ATRA, although only 27 are unquestionably regulated by ATRA via RAR-RXR heterodimers bound to RAREs (Balmer *et al.*, 2002). Many of these genes are involved with retinoid signalling pathways, thus ATRA induces the transcription of RARα2 (Petkovich *et al.*, 1987), RARβ2 (Brand *et al.*, 1988), RARγ2 (Lehmann *et al.*, 1992) and CRABP-II (Astrom *et al.*, 1991). Interestingly, a number of neuronal genes are known to be directly regulated by ATRA (reviewed in (Lane *et al.*, 2005)), with verified RAREs found in the promoter region of genes for the D2 dopamine receptor (D2DR) (Samad *et al.*, 1997), monoamine oxidase B (Wu *et al.*, 2009), oxytocin (Richard *et al.*, 1991), gonadotropin-releasing hormone (Cho *et al.*, 1998) and neurogranin genes (Iniguez *et al.*, 1994). ATRA upregulates the transcription of the majority of ATRA-sensitive neuronal genes, with the notable exceptions being tyrosine hydroxylase, dopamine-β-hydroxylase and gonadotropin-releasing hormone (see (Lane *et al.*, 2005)).

Additionally, there is *in vitro* evidence to suggest neuronal genes with monoaminergic roles can be regulated by ATRA that include the NA transporter (Matsuoka *et al.*, 1997), tyrosine hydroxylase (Kobayashi *et al.*, 1994), dopamine β-hydroxylase (Cervini *et al.*, 1994) and the 5-hydroxytryptamine 1A receptor (5-HT_{1A}R) (Charest *et al.*, 1993). Given the role monoamines are thought to have in depression pathology (discussed later), these findings represent a possible association between retinoids and depression. However, in all cases, the required promoter and sequence analyses have not

been performed and so it is unclear whether these genes are directly transcriptionally controlled by ATRA. All neuronal genes with verified RAREs or neuronal genes putatively thought to be influenced by ATRA are summarised in Table 1.1.

1.2.3. Functional roles of retinoids in the CNS

Early studies led to the discovery of ATRA as a morphogen in developing vertebrate embryos (Thaller *et al.*, 1987), while excessive consumption of vitamin A was shown to be teratogenic (Wilson *et al.*, 1953). ATRA functions in the anteroposterior and dorsoventral patterning of the neural tube and plate, particularly the organization of the posterior hindbrain and the anterior spinal cord and is involved in the neuronal differentiation of neurons and glia via transcription factor genes (reviewed by (Maden, 2007)). However, as highlighted earlier (Chapter 1.2.2.2.), components of the retinoid signalling pathway-including metabolic enzymes, binding proteins and receptors are present in the mature brain and are increasingly viewed as being of physiological importance in the CNS (Lane *et al.*, 2005; Mey *et al.*, 2004).

Retinoid signalling components such as RARα, RXRβ and RALDH2 were found to be abundant in the adult hippocampus (Wagner *et al.*, 2002; Zetterstrom *et al.*, 1999) and this has led to the intensive study of the physiological role of retinoids in this brain region. Memory and learning are important hippocampal functions that are thought to be based on changes in synaptic efficacy in the form of adult long-term potentiation (Bliss *et al.*, 1993) and long-term depression (Collingridge *et al.*, 2010). Studies with RARβ null mice have shown that hippocampal CA1 long-term potentiation and long-term depression are eliminated alongside spatial memory and learning deficits (Chiang *et al.*, 1998). A later study found reduced long-term potentiation and long-term depression after 12 weeks of vitamin A deprivation in adult mice, and long-term depression was completely abolished after 15 weeks (Misner *et al.*, 2001). Furthermore, aged mice (21 months old) exhibit diminished hippocampal long-term potentiation alongside reduced expression of RARβ, RXRβ/γ and neurogranin mRNA in the whole brain compared with adult mice (4 months old). The functional effects could be reversed by

Gene	RARE analysis or ATRA treatment	Cell system	Assay employed	References
D2 dopamine receptor (D2DR)	Verified RARE	Striatum	mRNA, protein	Samad *et al*., 1997; Valdenaire *et al*., 1998
Monoamine oxidase B (MAOB)	Verified RARE	BE(2)C cells	mRNA, function	Wu *et al*., 2009
Oxytocin	Verified RARE	Neuro2A	mRNA	Richard *et al*., 1991
Neurogranin	Verified RARE	SK-N-BE, striatum	mRNA	Husson *et al*., 2004; Iniguez et al., 1994
Gonadotropin-releasing hormone	Verified RARE	GT1-1, hypothalamus	mRNA	Cho *et al*., 1998; Cho *et al*., 2001
5-HT_{1A} receptor	ATRA increases expression	SN-48	mRNA	Charest *et al*., 1993
Dopamine D1, D2, D5 receptors	ATRA increases expression	NT2	mRNA, function	Sodja *et al*., 2002
NA transporter	ATRA increases expression	PC12, SCG	mRNA, function	Matsuoka *et al*., 1997
Vesicular acetylcholine transporter	ATRA increases expression	PC12, SN56, NG108	mRNA	Berse *et al*., 1995; Berse *et al*., 1997; Dolezal *et al*., 2001
Vesicular GABA transporter	ATRA increases expression	PC19	mRNA	Ebihara *et al*., 2003
Choline acetyltransferase	ATRA increases expression	PC12, SN56	mRNA, protein, activity	Berse *et al*., 1995; Berse *et al*., 1997; Personett *et al*., 2000
Tyrosine hydroxylase	ATRA decreases expression	SCG	mRNA, activity	Kobayashi *et al*., 1994

Dopamine β-hydroxylase	ATRA decreases expression	SCG	Activity	Berrard *et al*., 1993
Glutamic acid decarboxylase	ATRA increases expression	P19, NE-7C2	mRNA	Bain *et al*., 1993; Varju *et al*., 2002
Acetylcholine esterase	ATRA increases expression	P19	mRNA	Coleman *et al*., 1996
Mu opioid receptors	ATRA increases/decreases expression	SH-SY5Y	mRNA	Jenab *et al*., 2002
Delta opioid receptor	ATRA increases expression	NG108-15	mRNA	Beczkowska *et al*., 1996
Kappa opioid receptor	ATRA decreases expression	P19	mRNA	Bi *et al*., 2001
Nicotinic acetylcholine receptor α3,α4, β2	ATRA increases expression	P19	mRNA, protein	Cauley *et al*., 1996
NMDA receptor (NR1 subunit)	ATRA increases expression	NG108-15	mRNA	Beczkowska *et al*., 1996
Kainate receptor (GluR6 subunit)	ATRA increases expression	P19	mRNA	Bain *et al*., 1996
$GABA_A$ receptor γ2	ATRA increases expression	P19	mRNA, function	Reynolds *et al*., 1996

Table 1.1: Summary of neuronal genes regulated by retinoids. The top row consists of five neuronal genes with verified RAREs in the promoter region as determined by promoter or sequence studies. Additionally, other neuronal genes sensitive to ATRA treatment have been studied, although the presence of a RARE has not been tested or confirmed. The cell lines used were: BE(2)C = human neuroblastoma cell line, Neuro2A = murine neuroblastoma cell line, SK-N-BE = human neuroblastoma cell line, GT1-1= mouse-derived hypothalamic GnRH neuronal cells, PC12 = rat pheochromocytoma cells, SCG = rat superior cervical ganglia neurons, SN-48 = murine septum x neuroblastoma fusion cell line, SN-56= murine cholinergic cell line from septum, SH-SY5Y = human neuroblastoma cell line, NG108-15 = mouse neuroblastoma X rat glioma hybrid cell line, P19 = embryonal carcinoma cells and NE-7C2 = mouse p53-deficient neuroectodermal cell-line. Table modified from Lane *et al*., 2005.

vitamin A supplements (Etchamendy *et al.*, 2003). The hippocampus is also the site of adult neurogenesis: a process that involves the proliferation, differentiation and integration of new neurons into the mature hippocampus (Kempermann *et al.*, 2004). Deficits in adult neurogenesis are thought to contribute to depression (as discussed in Chapter 1.3.1.6.). There is growing evidence that ATRA is involved in hippocampal neurogenesis, given that vitamin A deficient adult mice have decreased levels of neuronal differentiation within the granular cell layer of the dentate gyrus (Jacobs *et al.*, 2006).

Beyond the hippocampus, retinoid signalling components including RALDH, RARs and CRABP-II, are colocalised with dopaminergic neurons originating from the substantia nigra and ventral tegmental area (VTA) and their forebrain projections into the striatum, the frontal cortex and the limbic system (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). ATRA can affect dopaminergic gene expression, while the retinoid receptor knockout mice RARβ-RXRβ$^{-/-}$, RARβ-RXRγ$^{-/-}$ and RXRβ-RXRγ$^{-/-}$ demonstrate a locomotor deficit accompanied by decreased D2DR expression (Krezel *et al.*, 1998).

Some of the functional roles of retinoid signalling in the adult CNS have been uncovered in the context of neurological diseases such as Alzheimer's disease (Goodman, 2006) and Huntington's disease (Mey *et al.*, 2004). Amyloid plaque formation has generally been viewed as a causal factor of Alzheimer's disease (Glenner *et al.*, 1984; LaFerla *et al.*, 2007) and studies show molecular components of the plaque formation cascade may be regulated by ATRA. The *in vitro* mRNA expression of the amyloid plaque precursor was shown to be increased following the application of ATRA (Konig *et al.*, 1990), although a definitive RARE has not yet been reported (Yang *et al.*, 1998). Additionally, ATRA is known to upregulate presenilin 1and 2 mRNA *in vitro*, that may promote amyloid plaque formation (Hong *et al.*, 1999) and regulates the expression of a major susceptibility gene in Alzheimer's disease called apolipoprotein E (Cedazo-Minguez *et al.*, 2001; Harris *et al.*, 2004). Evidence for the role of retinoids in Huntington's disease comes from a DNA array study that demonstrated the down-regulation of genes with RARES, including the D2DR, RBP and RARγ, in the R6/2 Huntington's disease mouse model (Luthi-Carter *et al.*, 2000).

Schizophrenia (Goodman, 1998; LaMantia, 1999) and depression (Bremner *et al.*, 2007) have also been linked with retinoid dysregulation which suggests that intact retinoid signalling may be required for normal functioning of the adult CNS. The association between retinoid signalling and schizophrenia derives from the similarity between the symptoms of retinoid toxicity/deficiency and schizophrenia, which include mental deficits and congenital malformations (reviewed in (Goodman, 1996)). Additionally, there is a convergence of the retinoid signalling loci such as the RARα, RARβ, RXRβ and RXRγ and schizophrenia candidate genes (reviewed in (Goodman, 1998)). Thirdly, a plethora of candidate genes for schizophrenia are targets of ATRA transcriptional regulation including the D2DR (Arinami *et al.*, 1997; Samad *et al.*, 1997). A number of lines of evidence implicate retinoid signalling with depression pathology and this is described in further detail in the following chapter below.

1.3. Retinoid-induced depression

Emerging research has implicated retinoids in causing or precipitating depression-related events such as depression, suicide ideation and completed suicide. The evidence is controversial and at times contentious, due to the small number of studies undertaken, difficulty in interpreting human patient data and the limitations in our understanding of depression pathology.

1.3.1. Major depression

Depression is viewed as a complex disorder that is both biologically and genetically heterogenous and is able to manifest itself at psychological, behavioural and physiological levels (aan het Rot *et al.*, 2009; Levinson, 2006; Wong *et al.*, 2001). Some of the complexity derives from the multitude of symptoms that can characterise clinical depression and its comorbidity with illnesses such as anxiety. The symptoms, also known as endophenotypes, listed in the Diagnostic and Statistical Manual IV (American Psychiatric Association, 1994) and the International Classification of Diseases (World Health Organisation, 1992) include depressed mood (or increased irritability), diminished interest or

pleasure (often described as anhedonia), increase/decrease in appetite, insomnia or hypersomnia, fatigue or loss of energy, psychomotor agitation or retardation, indecisiveness or inability to concentrate, feelings of worthlessness and suicide ideation. All but the most severe symptoms are not qualitatively different from those that many experience frequently in their lives, although diagnosis of clinical depression generally requires the presence of at least five symptoms over a two week period (American Psychiatric Association, 1994). In addition, there exists a number of further subdivisions within major depression including the onset (early, postpartum, late), clinical course (single, recurrent, chronic), severity (from mild to severe), presence or absence of psychotic symptoms, presence or absence of catatonic symptoms, seasonal pattern, whether it is secondary to illness and many others (Wong *et al.*, 2001). All aspects of depression are based on subjective descriptions of the symptoms and it is unknown whether they derive from differing biological mechanisms or are purely different manifestations stemming from a singular biological process.

Worldwide estimates of the lifetime prevalence of depression range from 4% to 10 % (Waraich *et al.*, 2004), with large regional variations (eg. 0.8% in Taiwan and 5.8% in New Zealand (Wong *et al.*, 2001)). The estimated point prevalence for a depressive episode among 16 to 74-year-olds in the UK in 2000 was 2.6% (males 2.3%, females 2.8%) (National Institute for Health and Clinical Excellence (NICE), 2009; Singleton *et al.*, 2001). Meanwhile, the incidence of the broader and less specific category 'mixed depression and anxiety' was found to be 11.4% (males 9.1%, females 13.6%).

Adolescence, a time of great social and neuroanatomical development (Spear, 2000), is itself a risk factor for depression. The prevalence for depression rises during early adolescence and is thought to affect 17%–25% of the late adolescent population (Kessler *et al.*, 2001). Depression that emerges during adolescence is typically episodic in nature, with episodes lasting 7-9 months (Emslie *et al.*, 2005a), in addition to being more chronic, severe and an increased number of suicide attempts compared with adult-onset of depression (Zisook *et al.*, 2007). Adolescence is characterised by the overproduction of synapses and receptors within most brains regions and their subsequent elimination (Andersen, 2003; Giedd *et al.*, 1999). These high levels of neuronal remodelling in adolescents create

windows of vulnerability whereby environmental factors, such as stress, may increase the susceptibility to depression (Andersen *et al.*, 2004).

Epidemiological studies have demonstrated that depression has approximately 31-42% heritability, although some estimates are considerably higher (Sullivan *et al.*, 2000). Only a few genes that confer risk have been identified (Canli *et al.*, 2007; Levinson, 2006)), but are assumed to be numerous and likely to interact with other non-genetic, or environmental (Caspi *et al.*, 2003), factors making the disorder both multigenetic and multifactorial. Currently no single genetic or environmental factor can account for more than 5% of the variance between depressed and normal subjects (Mann *et al.*, 2006). Environmental factors affecting depression susceptibility may include stressful life events often in the form of early childhood trauma such as neglect, physical or sexual abuse and parental loss (Heim *et al.*, 2001). There has been some debate as to whether stress is simply an epiphenomenon of depressed mood rather than a causal factor (Chrousos *et al.*, 1992), although a number of studies have demonstrated a potential pathophysiological role of stress systems in depression (Brady *et al.*, 1992; Habib *et al.*, 2000; Wong *et al.*, 2000).

The biological mechanisms behind depression aetiology have been investigated intensively and include the monoamine hypothesis that encompasses the serotonergic, dopaminergic and noradrenergic pathways, stress and the hypothalamic-pituitary-adrenal axis, neurogenic mechanisms and others. All mechanisms have both supportive and contradictory evidence and no singular mechanism is thought to underlie all the facets of depression pathophysiology. In fact, it has been suggested that there are a number of overlapping mechanisms in depression, with each mechanism specific to a subset of depression (Belmaker *et al.*, 2008).

1.3.1.1. Monoamine hypothesis

The monoamine hypothesis (also known as the biogenic monoamine hypothesis) postulates that depression arises through a depletion or imbalance of the monoamine neurotransmitters known as

noradrenaline (NA), serotonin (5-HT) and dopamine (DA) in the CNS (Owens *et al.*, 1998; Ressler *et al.*, 1999). Both NA and DA are synthesised through a common pathway from the precursor tyrosine (Figure 1.5.). The monoamine serotonin (5-hydroxytryptamine or 5-HT) is synthesised from tryptophan, which is converted inside the nerve terminal to 5-hydroxytryptophan (5-HTP) by the rate-limiting enzyme tryptophan hydroxylase (TPH).

Upon release, monoamines are able to bind to an array of specific receptors on both presynaptic and postsynaptic terminal membranes. In the case of NA, it is able to mediate noradrenergic responses by binding to three families of adrenergic receptors: α1, α2 and β. Serotonin is able to bind to 7 main types of 5-HT receptors (1–7), comprising a total of 14 structurally and pharmacologically distinct mammalian 5-HT receptor subtypes (Barnes *et al.*, 1999). Meanwhile DA is able to bind to D1-like receptors that includes the D1 dopamine receptor (D1DR) and D5DR or to D2-like receptors that include the D2DR, D3DR and D4DR (Missale *et al.*, 1998; Neve *et al.*, 2004).

The actions of all monoamines are terminated by the active reuptake of the monoamines into the presynaptic neuron by Na^+/Cl^- dependent transporters (Nelson, 1998). Back in the nerve terminal, monoamines can be metabolised by two isoforms of monoamine oxidase (MAO) known as MAOA and MAOB which preferentially bind to 5-HT/NA and β-phenylethylamine/benzylamine respectively (DA and tryptamine are metabolised equally by both isoforms). Meanwhile, catechol-O-methyltransferase (COMT) metabolises DA, NA and adrenaline.

The monoamine hypothesis arose through the chance finding that iproniazid, a compound originally intended for the treatment of tuberculosis, could elevate mood in depressed patients (Lopez-Munoz *et al.*, 2009). The mechanism behind this was subsequently discovered to be the inhibition of the enzyme monoamine oxidase (Delay *et al.*, 1952) which resulted in increased postsynaptic stimulation through increased neurotransmitter availability. Similarly, imipramine was accidentally discovered to alleviate depression in schizophrenic patients (Kuhn, 1958) and follow-up pharmacological studies using

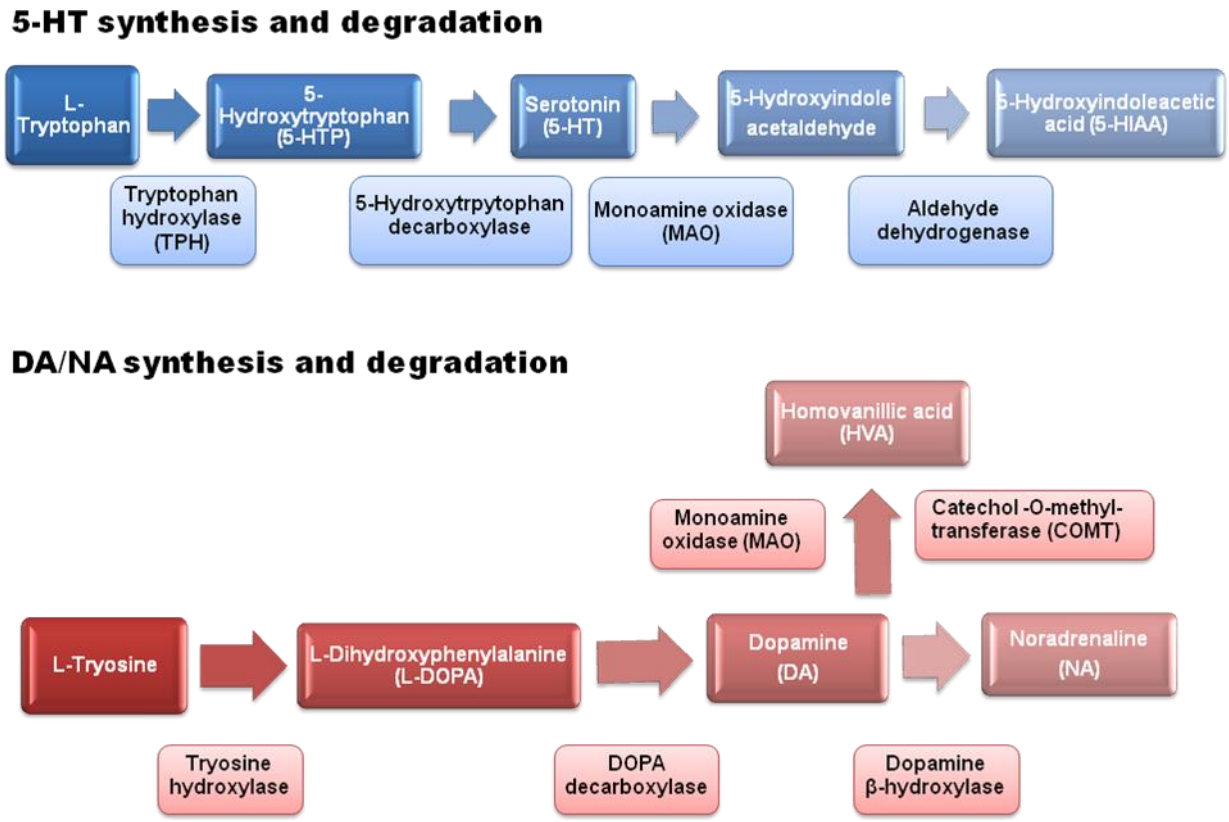

Figure 1.5: Monoamine biosynthesis and degradation pathways. Serotonin is synthesised in a two step process, with the conversion of L-tryptophan to 5-HTP by the TPH enzyme, followed by the conversion of 5-HTP to serotonin by the 5-hydroxytryptophan decarboxylase enzyme. Serotonin is subsequently metabolised by MAO into 5-hydroxyindole acetaldehyde, followed by conversion to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase. DA is synthesised by the conversion of L-tryosine into L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase, followed by the conversion of L-dihydroxyphenylalanine into DA by DOPA decarboxylase. DA is degraded by both MAO and COMT, creating homovanillic acid (HVA). NA is synthesised from DA by the dopamine- β-hydroxylase enzyme.

cardiovascular preparations, led to the seminal concept that imipramine (and its metabolite desipramine) owed their clinical efficacy to the inhibition of monoamine uptake and, in particular, NA (Gillette *et al.*, 1961). Further studies revealed the ability of imipramine and its tertiary amine derivatives clomipramine and amitriptyline to inhibit 5-HT reuptake more effectively than NA both *in vitro* and *in vivo* (Carlsson, 1970; Carlsson *et al.*, 1968; Lidbrink *et al.*, 1971). This led to the idea that inhibition of 5-HT uptake was responsible for the mood elevating effects of tertiary amine tricylic antidepressants (TCAs), whereas secondary amine TCAs were more potent at blocking NA uptake

(Carlsson *et al.*, 1969; Carlsson *et al.*, 1966) and the eventual development of serotonin selective reuptake inhibitors (SSRIs) such as fluoxetine (Wong *et al.*, 2005).

Other evidence of 5-HT involvement in depression aetiology came from studies showing that there were reductions in 5-HT levels and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in autopsy samples of the hind-brain of depressed suicide patients compared with those dying of sudden death or coronary occlusion (Bourne *et al.*, 1968; Shaw *et al.*, 1967). In addition, depression in human patients could be induced following treatment with an antihypertensive drug known as reserpine, which depletes both catecholamines and 5-HT (Goodwin *et al.*, 1971), whereas treatment with parachlorophenylalanine, a drug that depletes central 5-HT by inhibiting TPH, blocks the beneficial effects of TCAs and monoamine oxidase inhibitors (MAOIs) (Shopsin *et al.*, 1976; Shopsin *et al.*, 1975). Indirect biochemical measurements demonstrated 5-HT abnormalities in patients with major depression such as reduced uptake of 5-HT in blood platelets (Coppen *et al.*, 1978), reduction of 5-HIAA levels in the cerebrospinal fluid (CSF) (Asberg *et al.*, 1976; Reddy *et al.*, 1992), diminished prolactin response to acute 5-HT reuptake inhibitor challenge (Cowen *et al.*, 1987; Siever *et al.*, 1984) and a decrease in tryptophan, the precursor to 5-HT, in plasma (Coppen *et al.*, 1973; Cowen *et al.*, 1989). However, tryptophan depletion in healthy volunteers was shown to have no effect on depression despite reducing plasma tryptophan and 5-HT synthesis although it did cause relapse of depression of patients previously treated with antidepressants (reviewed in (Bell *et al.*, 2001)).

Alongside 5-HT, other studies confirm the role of NA in depression. For example, the administration of a tyrosine hydroxylase inhibitor called α-methyl- para-tyrosine that causes catecholamine (DA and NA) depletion induces relapse in patients who have been treated successfully with a NA reuptake inhibitor (Booij *et al.*, 2003), although it does not induce depression in normal subjects.

While the monoamine hypothesis remains the most researched and established mechanism for depression pathology, the hypothesis remains plagued with discrepancies and unresolved issues. For example, tianeptine enhances serotonin reuptake, an action that directly opposes that of SSRIs, yet is a

highly efficacious antidepressant (Uzbay, 2007). Attempts to induce depression through acute tryptophan depletion, which transiently lowers 5-HT brain activity through dietary restriction, has been shown to have no effect on healthy volunteers (Ruhe *et al.*, 2007). Perhaps the largest problem with the monoamine hypothesis is the temporal delay between the increase of monoamines at the synapse caused by antidepressant administration (within hours) and the onset of observable therapeutic improvements in patients (weeks or months of continuous administration) (Baldessarini, 1989). This suggests that acute elevation of monoamines cannot explain the whole mechanism of antidepressant action and likewise, does not provide a full understanding of the pathophysiology of depression. More recent research has moved beyond the measurement of global monoamine levels, but rather to focus on individual molecular components of monoaminergic signalling pathways that include receptors, enzymes and transporters.

1.3.1.2. The role of serotonergic pathways and components

The serotonergic pathways arise from the brainstem raphe nuclei that are found lying in or lateral to the midline regions of the pons and upper brainstem (Jacobs *et al.*, 1992). The raphe nuclei can be broadly divided into the caudal linear nucleus, dorsal raphe nucleus (DRN), the median raphe nucleus (MRN) and supralemniscal region (Pineyro *et al.*, 1999). The DRN is the largest of the brainstem serotonergic nuclei containing about 50– 60% of 5-HT neurons in the human CNS and innervates cortical regions and the neostriatum (Baker *et al.*, 1990; Descarries *et al.*, 1982). Meanwhile the MRN forms the second largest cluster of 5-HT neurons in the mammalian CNS and innervates the limbic system (Baker *et al.*, 1990).

1.3.1.2.1. 5-HT_{1A}R

Increasing attention has been placed on the role of the 1A subtype of 5- HT receptors (5-HT_{1A}R) in depression pathology (Savitz *et al.*, 2009). The 5-HT_{1A}R is a seven transmembrane G protein coupled receptor, which inhibits adenyl cyclase via Gα proteins (Raymond *et al.*, 2001), and in particular $G\alpha_i$ and $G\alpha_o$ subunits (Raymond *et al.*, 1993), thereby reducing levels of cyclic adenosine monophosphate

(Bockaert *et al.*, 1987). The 5-HT_{1A}R is one of the most abundant 5-HT receptor subtypes in the mammalian brain (Barnes *et al.*, 1999) and are widely expressed somatodendritically (between the soma and dendritic branches) within the DRN (Sotelo *et al.*, 1990) and postsynaptically on pyramidal cells and interneurons of the cortex, hippocampus, septum, amygdala and hypothalamus (Hensler *et al.*, 1991). The stimulation of postsynaptic 5-HT_{1A}Rs (either by 5-HT or 5-HT_{1A} agonists) is inhibitory on glutamatergic neurons (Sprouse *et al.*, 1988). Meanwhile, the activation of the somatodendritic 5-HT_{1A}Rs in the DRN is able to reduce the firing rate of these neurons, the amount of 5-HT released per action potential, the synthesis of 5-HT and therefore, the serotonergic activity to projection areas (Blier *et al.*, 1987; Hjorth *et al.*, 1991; Hutson *et al.*, 1989; Kreiss *et al.*, 1994; Meller *et al.*, 1990; Sprouse *et al.*, 1986; Verge *et al.*, 1985; Wang *et al.*, 1977).

Evidence of the involvement of 5-HT_{1A}Rs in depression pathology comes from a number of human post-mortem and polymorphism studies, in addition to reports from 5-HT_{1A}R knockout mice. Analysis of depressed human patients post mortem has revealed reduced 5-HT_{1A}R ligand binding in the ventrolateral prefrontal cortex and the temporal cortex as determined by autoradiography studies (Bowen *et al.*, 1989), reduced 5-HT_{1A}R ligand binding in the caudal aspects of the dorsal raphe nucleus (Arango *et al.*, 2001) and a reduction in 5-HT_{1A}R mRNA expression in the dorsolateral prefrontal cortex and hippocampus (Lopez-Figueroa *et al.*, 2004). Reduced 5-HT_{1A}R expression may reflect a compensatory mechanism in response to the hyposerotonergic state present in depressed patients. Conversely, a number of studies have shown an increase in 5-HT_{1A}R ligand binding such as the rostral regions of the raphe of depressed patients (Arango *et al.*, 2001; Boldrini *et al.*, 2008). Moreover, imaging studies that have employed PET technology with the potent 5-HT_{1A}R antagonist known as [11C]WAY-10063 have demonstrated a reduction of binding potential of 5-HT_{1A}R in the raphe and limbic regions of depressed patients (Drevets *et al.*, 2000; Sargent *et al.*, 2000) and the hippocampus, raphe nuclei, cingulate cortex and amygdala of depressed non-human primates (Shively *et al.*, 2006).

A number of human polymorphisms have been identified in the 5-HT_{1A}R gene (Arias *et al.*, 2002; Erdmann *et al.*, 1995; Kawanishi *et al.*, 1998; Nakhai *et al.*, 1995), although no clear association with depression has been established. However, the C(-1019)G single nucleotide polymorphism (SNP) found in the promoter of 5-HT_{1A}R has been associated with major depression in human patients (Lemonde *et al.*, 2003; Wu *et al.*, 1999). In these studies, the G allele was found to be twofold higher in patients with major depression and four times higher in completed suicides compared with control patients.

The knockout of 5-HT_{1A}R in mice has been widely shown to induce an anxious phenotype such as reduced exploratory behaviour and enhanced reactivity to fear cues (Heisler *et al.*, 1998; Parks *et al.*, 1998; Ramboz *et al.*, 1998). Furthermore, 5-HT_{1A}R KO mice exhibit increased immobility times in the tail suspension test compared with wildtype mice and could not be ameliorated by paroxetine and fluoxetine (Mayorga *et al.*, 2001). However, consistent with its role as an inhibitory autoreceptor, the genetic knockout of 5-HT_{1A}R in mice leads to immediate increases of 5-HT release in response to fluoxetine compared with wild type mice (He *et al.*, 2001).

Furthermore, the somatodendritic 5-HT_{1A} autoreceptors may play a role in antidepressant responses, whereby they contribute to the delay between commencement of antidepressant treatment and the therapeutic benefits observed two to three weeks later (Albert *et al.*, 1996; Albert *et al.*, 2004). The commencement of SSRI and TCA treatment elevates 5-HT levels but is immediately compensated by 5-HT_{1A} autoreceptor activation, thereby reducing raphe neuronal firing and 5-HT release (Hjorth *et al.*, 2000; Stahl, 1998). However, chronic SSRI treatment is known to induce a progressive functional desensitisation of 5-HT_{1A}Rs in animals (Blier *et al.*, 1990; Le Poul *et al.*, 2000) potentially leading to the disinhibition of 5-HT neuronal firing, thereby enhancing 5-HT release. The functional desensitisation of 5-HT_{1A}Rs via chronic antidepressant treatment might occur through the specific internalization and loss of the 5-HT_{1A} autoreceptors, but not postsynaptic receptors (Albert *et al.*, 2004; Hervas *et al.*, 2001; Riad *et al.*, 2001), although no differences in 5-HT_{1A}R density have also been previously reported (Jolas *et al.*, 1994; Le Poul *et al.*, 2000).

1.3.1.2.2. SERT

The 5-HT reuptake transporter (5-HTT, SERT) is inextricably linked with antidepressant action (Owens *et al.*, 1994) and has been extensively studied in depression research. SERT is a 12-transmembrane domain protein located along nerve processes and nerve terminals (Langer *et al.*, 1980) and its role is the termination of the action of 5-HT after it is released from the nerve terminal. SERT is located on the presynaptic neuron and takes up one 5-HT molecule concurrently with one Na^+ ion, decreasing extracellular concentrations of 5-HT to levels where postsynaptic receptor activation ceases (Purselle *et al.*, 2003). SSRIs enhance serotonergic neurotransmission by blocking the 5-HT-binding site of SERT, thus preventing 5-HT uptake into the neuron (Backstrom *et al.*, 1989; Graham *et al.*, 1992; Owens *et al.*, 1994). Similar to the 5-HT_{1A}R, SERT may be involved in the delayed therapeutic effects of antidepressants because chronic SSRI treatment was shown to cause downregulation of SERT expression *in vivo* and *in vitro* (Benmansour *et al.*, 1999; Benmansour *et al.*, 2002; Horschitz *et al.*, 2001; Pineyro *et al.*, 1994), although others have reported no such changes (Graham *et al.*, 1987; Kugaya *et al.*, 2003). The downregulation of SERT following chronic SSRI treatment is thought to derive from internalisation of SERT from the cell-surface membrane into the presynaptic neuron (Lau *et al.*, 2009; Lau *et al.*, 2008), thereby reducing extracellular 5-HT reuptake.

For over 20 years, a plethora of studies have sought to measure SERT ligand binding in post-mortem brain tissue of suicide victims (reviewed in (Purselle *et al.*, 2003)). Using mainly [3H]-imipramine and [3H]-paroxetine as radioligands, post-mortem studies reveal both increases (Arato *et al.*, 1991; Gross-Isseroff *et al.*, 1989) and decreases (Stanley *et al.*, 1982) in SERT ligand binding in depressed suicide victims. Despite the raphe nucleus containing the highest density of SERT, only two studies have focused on this structure and both were unable to show any significant changes in SERT ligand binding (Arango *et al.*, 2001; Bligh-Glover *et al.*, 2000). However single-photon emission-coupled tomography studies have shown a 19% reduction in the density of brainstem SERT binding sites (Malison *et al.*, 1998) and a 10% reduction in SERT binding potential in the midbrain (Joensuu *et al.*,

2007) of depressed patients compared with controls suggesting SERT availability may be altered in depression.

Human SNPs found within the SERT gene have been studied extensively and associated with depression (Murphy *et al.*, 2008; Neumeister *et al.*, 2004; Owens *et al.*, 1994). One polymorphism, known as 5-HTTLPR (Heils *et al.*, 1996; Lesch *et al.*, 1996), is a 44bp insertion (LL)/deletion (SS) polymorphism in the transcriptional control region of SERT, with the short form of this variant labelled 's' and a long form 'l' (Heils *et al.*, 1997). The short form has been associated with lower transcriptional efficiency and therefore lower serotonin uptake activity, compared with the long form. Counterintuitively, it is the short variant that some have argued that confers susceptibility for depression (Collier *et al.*, 1996), suicidal behaviour (Bellivier *et al.*, 2000; Courtet *et al.*, 2003) and a poorer response to SSRI antidepressant treatment (Yu *et al.*, 2002).

Meanwhile, transgenic mouse models have shown that $SERT^{-/-}$ mice exhibit depression-like and anxiety-like behaviour (Holmes *et al.*, 2003; Lira *et al.*, 2003). For instance, $SERT^{-/-}$ mice and rats display increased immobility in the FST (Holmes *et al.*, 2003; Olivier *et al.*, 2008) and $SERT^{-/-}$ mice spend a greater time immobile in the tail suspension test (Alexandre *et al.*, 2006). This paradoxical depressive effect of knocking out SERT may be explained by the observation that serotonergic cell number in the DRN was reduced by 50%, with a concomitant fourfold decrease in firing rate in $SERT^{-/-}$ mice compared with wildtype mice (Lira *et al.*, 2003). In addition, $SERT^{-/-}$ mice also have reduced 5-HT_{1A}R density in the hypothalamus, amygdala and dorsal raphe nucleus (Li *et al.*, 2004) that suggests an association between the receptor and transporter.

1.3.1.2.3. TPH2

TPH is the rate-limiting enzyme involved in the biosynthesis of 5-HT (see Figure 1.5) and a neuronal specific isoform known as TPH2 (Walther *et al.*, 2003b) has been linked to depression. The synthesis of 5-HT requires two enzymatic steps; firstly amino acid L-tryptophan is hydroxylated by the specific

enzyme TPH , with molecular oxygen and pterin as cosubstrates to create 5-HTP, followed by the decarboxylation of 5-HTP by the enzyme 5-hydroxytryptophan decarboxylase ((Hamon *et al.*, 1981) and Figure 1.5). The activity of TPH was found to be 70-100 times less than that of 5-hydroxytryptophan decarboxylase and indicated that the rate-limiting step of 5-HT synthesis was TPH (Hamon *et al.*, 1979). More recently, a neuronal specific isoform of TPH, known as TPH2, was identified and found to be responsible for brain 5-HT synthesis (whereas TPH1 is involved in peripheral 5-HT synthesis) (Walther *et al.*, 2003b). High levels of TPH2 are found in the serotonergic neurons of the raphe and to a lesser extent in forebrain regions such as the hippocampus, striatum and cortex (Gutknecht *et al.*, 2009).

The role of TPH2 in depression is demonstrated by human post-mortem studies that show depressed suicides have a 33% increase in TPH2 mRNA expression in the dorsal raphe nucleus (Bach-Mizrachi *et al.*, 2006) and depressed suicides have a greater density and number of TPH-immunoreactive neurons in the dorsal raphe nucleus (Bach-Mizrachi *et al.*, 2008; Underwood *et al.*, 1999). This apparently paradoxical increase may be a homeostatic response to reduced levels of 5-HT thought to occur in depressed patients or it may be that the isoenzyme form of TPH2 has reduced activity, thereby reducing 5-HT synthesis. It also appears as though chronic fluoxetine treatment can directly affect TPH2 levels in rats as demonstrated by two studies that show an initial reduction of TPH2 mRNA after two weeks (Dygalo *et al.*, 2006; Shishkina *et al.*, 2007) treatment, but subsequent increases in the midbrain following 4 and 8 weeks of treatment (Shishkina *et al.*, 2007).

Meanwhile, a number of groups have looked specifically at polymorphisms within the TPH2 gene (Waider *et al.*, 2011). A number of polymorphisms such as rs11178997 and rs4570625 are thought to affect the functionality of the TPH2 promoter in serotonergic cells (Chen *et al.*, 2008; Scheuch *et al.*, 2007), whereas the rs33849125 polymorphism leads to a loss of function of TPH2 in PC12 cells (Zhang *et al.*, 2005). In humans, a SNP (rs1386494 A/G) has been linked with major depression (Zill *et al.*, 2004), while the TPH2 -703G/T SNP is thought to effect the susceptibility to suicidal behaviour in depressed patients (Yoon *et al.*, 2009). Animal studies have likewise revealed an association

between TPH2 and depression pathology. For instance, the murine C1473G TPH2 gene polymorphism was associated with reduced TPH2 enzyme activity, reduced aggression levels and reduced immobility times in the FST (Osipova *et al.*, 2009). A second study also appears to show that male, but not female, $TPH2^{-/-}$ mice display reduced immobility in the FST (Savelieva *et al.*, 2008). However $TPH2^{-/-}$ mice displayed increases in immobility as measured by the tail suspension test. Knockin mice expressing a mutant form of TPH2 (equivalent to a rare human variant called R441H) have been created that display an 80% reduction in 5-HT synthesis and a significant increase in immobility time in the tail suspension test (Beaulieu *et al.*, 2008).

1.3.1.2.4. 5-HT_{1B}R and other 5-HT receptors

In addition to 5-HT_{1A}Rs, other 5-HT receptors have been implicated in depression such as the 5-HT_{1B}R (Sari, 2004) and 5-HT_{2A}R (Pandey *et al.*, 2002). Similar to the 5-HT_{1A}R, the 5-HT_{1B}R subtype belongs to the family of 5-HT_1 inhibitory Gα i/o receptors (Pedigo *et al.*, 1981) and have subsequently been shown to be homologous to the human 5-$HT_{1D\beta}$Rs (Adham *et al.*, 1992). The 5-HT_{1B}Rs are located on serotonergic neurons of the raphe nucleus (Doucet *et al.*, 1995) where they act as inhibitory autoreceptors negatively regulating 5-HT release (Sharp *et al.*, 1989; Starkey *et al.*, 1994) and controversially, may control serotonergic cell firing (Evrard *et al.*, 1999; Sprouse *et al.*, 1987). Further evidence of autoreceptor function comes from studies showing that the 5-HT_{1B}R agonist RU 24969 (Doods *et al.*, 1985) inhibits the release of 5-HT in the hippocampus (Martin *et al.*, 1992), frontal cortex (Sleight *et al.*, 1989) and diencephalon (Auerbach *et al.*, 1991). The 5-HT_{1B}Rs also exist as heteroreceptors on non-serotonergic receptors, such as the cholinergic terminals of the rat hippocampus and upon activation, inhibit the release of acetylcholine (Maura *et al.*, 1986).

The association between 5-HT_{1B}Rs and depression pathology is unclear based on human studies. One group reported significantly lower levels of 5-HT_{1B}Rs in the frontopolar cortex, orbitofrontal cortex (males only), hippocampus (females only) and higher levels in the paraventricular nucleus of suicide victims compared with healthy controls (Anisman *et al.*, 2008). However, an autoradiography study

found that 5-HT_{1B}R ligand binding in the prefrontal cortex of suicide victims with major depression was not different from healthy controls (Huang *et al.*, 1999) and another group similarly found no difference in 5-HT_{1B}R maximum binding, Bmax, or binding affinity, Kd, between the suicide and nonsuicide groups in the frontal cortex using autoradiography (Arranz *et al.*, 1994). Meanwhile, the frequency of two 5-HT_{1B}R polymorphisms (G861C and C129T) in patients with a history of major depression was shown to be not significantly different from controls (Huang *et al.*, 1999) and these findings appear to be corroborated by similar studies conducted in suicide victims (New *et al.*, 2001; Nishiguchi *et al.*, 2001).

Yet, animal studies have suggested a possible link between 5-HT_{1B}Rs and the mechanism of action of antidepressants. Studies have shown that chronic SSRI treatment can down-regulate and/or desensitize 5-HT_{1B} receptors in rats (Blier *et al.*, 1988; O'Connor *et al.*, 1994). In line with this, chronic treatment with fluoxetine was shown to reduce 5-HT_{1B} mRNA in the rat dorsal raphe nuclei and could be reversed by discontinuation of treatment (Neumaier *et al.*, 1996). Moreover, the ability of fluoxetine and paroxetine to increase 5-HT levels (in the frontal cortex and dorsal raphe nucleus of rats, respectively) appears to be potentiated by pretreatment with 5-HT_{1B}R antagonist GR 127935 (Davidson *et al.*, 1995; Gobert *et al.*, 1997). Similarly, the ability of fluoxetine to raise 5-HT levels mice was augmented in the hippocampus in 5-$HT_{1B}R^{-/-}$, although not in the striatum (Knobelman *et al.*, 2001).

Meanwhile , the 5-HT_{2A}R subtype, found predominantly on 5-HT receptive postsynaptic neurons of the cerebral cortex , is believed to be important in the context of depression aetiology (reviewed in (Elhwuegi, 2004)). Post-mortem studies have revealed a greater number of 5-HT_{2A} receptors in the prefrontal cortex in parallel with increased protein and mRNA expression in both the prefrontal cortex and hippocampus of young suicide victims (Pandey *et al.*, 2002) and an increased number of 5-HT_{2A}R binding sites in adult suicide victims (Hrdina *et al.*, 1993), although not all studies are in agreement (reviewed in (Arango *et al.*, 1997)).

In summary, there are a number of lines of evidence that suggest serotonergic components such as 5-HT_{1A}R, TPH2 and SERT are altered in depression. However, there are still questions with regard to whether these alterations are causal factors in depression aetiology or the downstream results of other neuronal changes taking place. Alongside 5-HT, a considerable amount of research has focused on understanding the roles of NA and DA in depression pathology.

1.3.1.3. The role of noradrenergic pathways and components

Most noradrenergic neurons are primarily located in the locus coeruleus of the brainstem, where projections innervate most of the cortical and subcortical areas in addition to the spinal cord, as well as the lateral tegmental portion of the reticular formation (Ressler *et al.*, 1999). NA release from the locus coeruleus has been shown to potentiate the firing of dopaminergic cells in the ventral tegmental area (Grenhoff *et al.*, 1993), while pharmacological activation of the adrenergic α1 and α2 receptors have opposing effects (increase and decrease, respectively) on the firing rate of DRN serotonergic neurons (Plaznik *et al.*, 1983).

1.3.1.3.1. Adrenergic receptors

All three families of adrenergic receptors (α1Rs, α2Rs and βRs) are seven transmembrane G protein-coupled receptors (GPCRs), although activation of each family of receptors leads to different downstream consequences. The α1 adrenergic receptors are typically excitatory in nature and may play a role in 5-HT firing in the raphe given that administration of α1 adrenergic receptor agonist phenylephrine stimulates 5-HT firing activity in the DRN and MRN (Judge *et al.*, 2006), whereas α1 adrenergic receptor antagonists suppress 5-HT neuron firing activity (Baraban *et al.*, 1980). In agreement with this trend, rats undergoing chronic treatment with a number of antidepressants were shown to have increased α1 binding (using [3H]prazosin as a ligand) in the cerebral cortex (Maj *et al.*, 1985).

In contrast, the α2 adrenergic heteroreceptors on serotonergic terminals are inhibitory in nature and regulate 5-HT release (Limberger *et al.*, 1986), while the role of α2 autoreceptors is to presynaptically regulate neurotransmitter release and has been implicated in the inhibitory control of adrenergic and serotonergic pathways innervating the frontal cortex (Dennis *et al.*, 1987; Limberger *et al.*, 1986). Furthermore, the activation of α2 adrenergic receptors has been shown to decrease NA output and suppresses the firing activity of 5-HT neurons in the dorsal raphe nucleus of rats (Clement *et al.*, 1992). A number of studies have shown that chronic desipramine treatment can result in hyporesponsive α2 receptors resulting in raised basal levels of extracellular NA in the dorsal hippocampus (Sacchetti *et al.*, 2001). Conversely, some studies suggest supersensitivity of the α2 receptor may be a predisposing factor for depression. Post-mortem studies of depressed suicide victims found an increased level of α2 adrenergic receptors in the prefrontal cortex compared with healthy controls (Garcia-Sevilla *et al.*, 1999).

1.3.1.3.2. COMT

The intracellular enzyme COMT, found mainly in postsynaptic dopaminergic neurons and glial cells (Karhunen *et al.*, 1995; Rivett *et al.*, 1983), is responsible for the *O*-methylation of DA and NA (Alexrod *et al.*, 1958), which inactivates the catecholamines (Mannisto *et al.*, 1999; Yavich *et al.*, 2007). The evidence of an association between COMT and depression derives from a number of human and animal studies. Erythrocyte COMT activity is significantly elevated in major depressive patients (Shulman *et al.*, 1978), while a COMT inhibitor, tolcapone, reverses anhedonia in a rat model of depression (Moreau *et al.*, 1994). The COMT gene contains a functional single-nucleotide polymorphism, rs4680, causing an amino acid substitution from valine to methionine (val158met, val allele associated with higher activity) and has been investigated for association with major depression with contradictory reports (Kunugi *et al.*, 1997; Massat *et al.*, 2005; Ohara *et al.*, 1998). However, it does appear to be linked with electroconvulsive therapy response (Domschke *et al.*, 2009), response to paroxetine treatment in major depressives (Benedetti *et al.*, 2009) and peripartum depression in combination with MAOA (Doornbos *et al.*, 2009).

1.3.1.4. The role of dopaminergic pathways and components

Depression is unlikely to be restricted to serotonergic and noradrenergic disturbances alone and increasing evidence suggests a relationship between alterations in DA pathways and depression (Nestler *et al.*, 2006). The main dopaminergic pathways within the CNS include *i)* the mesocortical pathway, *ii)* mesolimbic pathway (from the limbic area) which both originate from the ventral tegmental area and projects to the cortex, *iii)* the tuberoinfundibular pathway which originates from the hypothalamus and projects to the pituitary gland and *iv)* the nigrostriatal pathway that extends from the substantia nigra to the striatum (Dailly *et al.*, 2004). Within these pathways exist DA receptors that are divided in two subfamilies: the D1-like receptor subtypes (D1DR and D5DR) and the D2-like subfamily (D2DR, D3DR, and D4DR), with D1DR and D2DR present in the highest concentrations in the CNS (Missale *et al.*, 1998).

1.3.1.4.1. D2DR

The D2DR is a membrane Gα i/o-protein-coupled receptor that belongs to the family of D2-like DA receptors (including D3DRs and D4DRs) (Gingrich *et al.*, 1993). D2DRs are pharmacologically distinct from the D1-like DA receptors such as the D1DR (Seeman *et al.*, 1987b), although 'physiological antagonism' of the D2DR via the D1DR is widely observed, such that activation of neurons via the D1DRs is reduced by the concurrent activation of the D2DRs (Bonci *et al.*, 2005). The D2DR has two molecular isoforms known as D2Long and D2Short that arise through alternative splicing (Dal Toso *et al.*, 1989). It is thought that the D2Short isoform is the D2DR autoreceptor (Khan *et al.*, 1998), expressed in DA neurons, that regulates DA release, whereas the D2Long isoform functions postsynaptically as heteroreceptors on target cells exerting a variety of functions (Hopf *et al.*, 2003; Mottola *et al.*, 2002).

The D2DR has been implicated in depression pathology and antidepressant action from single-photon emission-computed tomography studies showing increased binding of the D2-like antagonist iodobenzamide in the basal ganglia of depressed patients (D'Haenen H *et al.*, 1994; Shah *et al.*, 1997)

and the antidepressant properties of the D2-like agonist bromocriptine in randomised control trials (Bouras *et al.*, 1982; Millan *et al.*, 2002). However, D2-like agonists and antagonists may also have activity at the D3 and D4 receptor subtypes, questioning the sole involvement of the D2DR subtype in these findings. Lower levels of DA and/or DA metabolites have been found in the serum and CSF of depressed patients (Engstrom *et al.*, 1999) that could both suggest a hypo-dopaminergic state. The anhedonic-like symptoms often seen in depression have been related to deficits in dopaminergic signalling in the mesolimbic pathway (Heinz *et al.*, 1994) while 20-40% of Parkinson's disease patients exhibit depression that may relate to altered mesolimbic and mesocortical pathways (Lieberman, 2006). However, a post-mortem study of depressed suicide victims found no alterations in D1DR and D2DR mRNA within the caudate nuclei (Hurd *et al.*, 1997).

Animal studies have similarly shown an association between the D2DR and depression pathology/antidepressant action. For instance, chronic treatment with imipramine, amitriptyline and mianserin treatment increased the binding activity of the D2-like agonist N-0437 in the limbic areas of the rat forebrain including the nucleus accumbens (Maj *et al.*, 1996). Similarly, 14 days of imipramine or mianserin treatment increased the binding of the D2-like agonist quinpirole (Maj *et al.*, 1998). Meanwhile, D2-like agonists such as pramipexole (Willner *et al.*, 1994) and quinpirole (Muscat *et al.*, 1992a) have antidepressive effects as demonstrated by the increased sucrose consumption of stressed and non-stressed rats. Conversely, the rescue of decreased sucrose consumption of rats (following the chronic mild stress paradigm (Muscat *et al.*, 1992a)) by chronic amitriptyline or desipramine could be blocked by acute administration of the D2-like receptor antagonist sulpiride (Sampson *et al.*, 1991). Similarly, the specific D2-like receptor antagonist raclopride blocked the rescue of decreased sucrose consumption of rats by chronic imipramine treatment (Muscat *et al.*, 1990). Furthermore, reduction of immobility times in the FST by desipramine, imipramine, or amitriptyline could be blocked by injection of the D2-like antagonist sulpiride in the nucleus accumbens (Cervo *et al.*, 1988) but not in the caudate-putamen (Cervo *et al.*, 1987).

1.3.1.4.2. MAOA

MAO is a flavin-adenine-dinucleotide-containing enzyme (Nara *et al.*, 1966) that exists as two isoenzymes, MAOA and MAOB, that are encoded by distinct genes (Bach *et al.*, 1988) and differ in terms of substrate preferences (Collins *et al.*, 1970), inhibitor specificities (Johnson, 1968) and cell/tissue distribution (Grimsby *et al.*, 1990). MAOA is localised around the mitochrondrial outer membrane and is involved in monoamine metabolism: it preferentially binds to 5-HT and DA, as well as tryptamine (Ma *et al.*, 2004).

Some early studies putatively linked MAOA in causing affective disorders (Brunner *et al.*, 1993; Deckert *et al.*, 1999) and a positron emission tomography (PET) study has shown that depressed patients have a 34% increase in MAOA density in many brain regions such as the prefrontal cortex, midbrain and hippocampus (Meyer *et al.*, 2006). Increased MAOA density could lead to the increased metabolism of 5-HT and DA, resulting in the lowered monoamine levels found in depressives. Additionally, men with a 30-bp variable number tandem repeat (VNTR) polymorphism in the promoter of MAOA (along with a dinucleotide repeat in intron 2) expressed lower serotonergic responsiveness in the fenfluramine challenge test and more impulsive aggression (Manuck *et al.*, 2000), although there appears to be no association with suicidality (Courtet *et al.*, 2005). The same polymorphism has additionally been linked with altered CSF 5-HIAA concentrations (Jonsson *et al.*, 2000), as well as major depression and bipolar disorder (reviewed in (Hattori *et al.*, 2005)). The role of MAOA in the pathology of depression in humans is further highlighted by the efficacy of MAOIs to treat depression (Riederer *et al.*, 2004), whereby phenelzine and tranylcypromine act by inhibiting both MAOA and MAOB, and brofaromine and moclobemide inhibit MAOA only (Papakostas, 2006). Meanwhile, in animals, the knockout of MAOA alters mouse behaviour in the form of increased aggression (Cases *et al.*, 1995).

1.3.1.5. 5-HT and DA interaction

There also exists considerable interaction between the DA and 5-HT signalling pathways (Alex *et al.*, 2007). This is thought to occur mainly through 5-HT_{2A}Rs (and 5-HT_{1A}Rs to a lesser extent) present on dopaminergic neurons in regions including the ventral tegmental area (Doherty *et al.*, 2000; Ikemoto *et al.*, 2000). For instance, administration of the selective 5-HT_{2A}R antagonist (MDL 100,907) was found to increase DA efflux in the rat prefrontal cortex (Schmidt *et al.*, 1995). It is therefore unsurprising that many psychiatric diseases including depression, bipolar depression and schizophrenia are thought to be caused by both 5-HT and DA alterations (Kahn *et al.*, 1993; Kosten *et al.*, 1998; Yatham *et al.*, 2005) and that some drugs such as antipsychotics bind to both 5-HT and DA receptors (Meltzer *et al.*, 1989). The findings suggest that 5-HT is capable of modulating the response of DA and could have implications for depression pathology.

1.3.1.6. Neurogenic theory of depression and other mechanisms

Adult neurogenesis is the term for the proliferation and functional integration of new neurons with existing neurons and occurs in two predominant areas: the subventricular zone lining the lateral ventricles and subgranular zone of the hippocampus (Lledo *et al.*, 2006). Adult neurogenesis is postulated to underlie the chronic adaptive neuronal processes of depression pathology and antidepressant action, as opposed to acute monoamine-mediated mechanisms (Castren *et al.*, 2007). All types of antidepressant treatment, including chronic fluoxetine administration (Malberg *et al.*, 2000) and electroconvulsive treatment (Madsen *et al.*, 2000), increase hippocampal neurogenesis in animal models. Furthermore, neurogenesis was demonstrated to be necessary for the anxiolytic effects of imipramine and fluoxetine in mice as measured by the novelty-suppressed feeding paradigm (Santarelli *et al.*, 2003) .

The link between neurogenesis and depression pathology may derive from alterations in neurotrophic factors such as brain derived neurotrophic factor (BDNF) (Castren *et al.*, 2007). A significantly lower level of BDNF was observed in the hippocampus of depressed suicide patients (Castren, 2004), while

increased BDNF expression was found in dentate gyrus and supragranular regions in patients treated with antidepressant medications at the time of death, compared with non-untreated patients (Chen *et al.*, 2001). A myriad of antidepressants such as citalopram and sertraline (Coppell *et al.*, 2003; Holoubek *et al.*, 2004), tranylcypromine (Russo-Neustadt *et al.*, 1999) and imipramine (Van Hoomissen *et al.*, 2003) can increase BDNF levels in the major subfields of the hippocampus, suggesting the neurogenic effects of antidepressants are mediated via BDNF.

Furthermore, 5-HT and BDNF are known to influence one another, sometimes acting in a cooperative manner (Mattson *et al.*, 2004). BDNF was reported to promote serotonergic neurotransmission, increasing the synthesis of 5-HT and the activity of serotonergic neurons (Siuciak *et al.*, 1998). Moreover, BDNF has been observed to promote axonal sprouting of 5-HT axons (Mamounas *et al.*, 2000). The interactions between BDNF and 5-HT signalling are thought to occur via the 5-HT_{1A}R, given that BDNF knockout mice displayed attenuation of 5-HT_{1A}R function in the hippocampus (Hensler *et al.*, 2007). Meanwhile antidepressant-induced upregulation of BDNF is attenuated by the 5-HT_{1A}R antagonist WAY-100635 (Ivy *et al.*, 2003) and conversely the 5-HT_{1A}R agonist 8-hydroxy-2(di-n-propylamino)tetralin (8-OH-DPAT) can increase neurogenesis (Banasr *et al.*, 2004). Finally, data has shown that 5-HT_{1A}R knockout mice treated chronically with fluoxetine do not display reduced anxiety-related behaviour as measured by the novelty-suppressed feeding paradigm (Santarelli *et al.*, 2003).

However some caveats exist within the neurogenic theory of depression. For example, the ablation of hippocampal neurogenesis via hippocampal-specific X-ray irradiation does not affect depression-related behaviour in animals, suggesting the inhibition of neurogenesis alone is insufficient to induce depression (Airan *et al.*, 2007). Conversely, infusion of BDNF does not produce antidepressant-like effects in all parts of the brain and produces an opposing depression-like phenotype when infused into the ventral tegmental area (Eisch *et al.*, 2003). Recently, the antidepressive effects of fluoxetine have been shown to be independent of neurogenesis (Holick *et al.*, 2008) and is in direct contrast with

previous findings that the anxioltyic effects of chronic fluoxetine treatment are neurogenesis-dependent (Santarelli *et al.*, 2003).

The activation of the hypothalamic-pituitary-adrenal axis is one of the prominent mechanisms through which the brain responds to stress and consists of neurons in the paraventricular nucleus of the hypothalamus that secrete corticotropin-releasing hormone (CRH) which in turn stimulates the synthesis and release of adrenocorticotropin from the anterior pituitary. Adrenocorticotropin then stimulates the synthesis and release of glucocorticoids from the adrenal cortex in the form of cortisol in humans and corticosterone in rodents (Berton *et al.*, 2006). Abnormal, excessive activation of the hypothalamic-pituitary-adrenal axis was observed in approximately half of individuals with depression and these abnormalities were corrected by antidepressant treatment (Arborelius *et al.*, 1999; Holsboer, 2001), while blockade of glucocorticod receptors have been shown to augment the antidepressive effects of fluoxetine (Johnson *et al.*, 2007; Johnson *et al.*, 2009). Glucocorticoids are also known to inhibit adult neurogenesis (Duman *et al.*, 2006), an effect that can be reversed by the glucocorticoid antagonist mifepristone (Oomen *et al.*, 2007), that further strengthens both the glucocorticoid and neurogenic theory of depression.

Although less well studied, other mechanisms thought to be involved with depression pathology include altered glutamatergic neurotransmission, reduced GABAergic (γ-aminobutyric acid) neurotransmission, abnormal circadian rhythms, deficient neurosteroid synthesis, impaired endogenous opioid function and cytokine mediated depression (reviewed in (Belmaker *et al.*, 2008).

1.3.2. Clinical studies of 13-*cis*-RA

1.3.2.1 Usage, mechanisms of action and pharmacokinetics

Synthetic retinoids were first chemically synthesised over 50 years ago and in 1955, the *trans* to *cis* transformation of ATRA resulted in the synthesis of its geometric isomer 13-*cis*-RA (O'Donnell, 2003), shown in Figure 1.1F. 13-*Cis*-RA was shown to be highly effective for the therapy of disorders

of keratinisation such as Darier disease, ichthyosis and cystic acne (Peck *et al.*, 1978) and by 1982, the Food and Drug Administration approved the use of 13-*cis*-RA (Tradenames: Accutane and Roaccutane) as an oral treatment for severe cystic or recalcitrant acne. Since its introduction in 1982 to 2000, 19.8 million prescriptions for Roaccutane were dispensed in the United States alone and the number is likely to rise given the trend for its use in milder forms of acne (Wysowski *et al.*, 2002).

Acne is caused by the interplay of the patient's skin bacteria and abnormal sebaceous lipids, as well as increased sebum production from sebocytes and ductal cornification (Cunliffe, 1998). Meanwhile the acne bacteria *Propionibacterium acnes* can colonise the pilosebaceous ducts, in the presence of comedones (blackheads and whiteheads), to form papules and possibly nodules. The mechanisms of action of 13-*cis*-RA are not completely understood, although it is thought to normalise the maturation and adhesion of keratinocytes thereby reducing comedone formation (Marcelo *et al.*, 1984) . It is also known to reduce sebocyte-mediated androgen synthesis (Torma, 2001), reduce sebum excretion (Strauss *et al.*, 1980) and reduce the number of *Propionibacterium acnes* (King *et al.*, 1982).

The efficiency of Roaccutane for the treatment of severe acne has been well established over the years and has been hailed as 'an incredible triumph…in the treatment of acne vulgaris' (Lowenstein, 2002). In most countries, the manufacturer's daily recommended dose is 0.5-1mg/kg taken orally (although doses are increased to 2mg/kg for non-responders), with the aim of a cumulative dose of 100-120mg/kg (Cunliffe *et al.*, 1997). A single course of 13-*cis*-RA for 15 to 20 weeks was shown to cause complete and prolonged remission of acne treatment (Farrell *et al.*, 1980; Jones *et al.*, 1980). In addition, 13-*cis*-RA was found to be more effective than either erythromycin antibiotic treatment (Zouboulis *et al.*, 2003) or the combination of oral tetracycline and topical retinoic acid (Langner *et al.*, 1985). After discontinuation of treatment, relapses in skin condition can occur in 20% of patients (Chivot *et al.*, 1990), although this number may be considerably higher (White *et al.*, 1998). The rates of patients with relapses in skin condition can be reduced by reaching higher cumulative doses of 13-*cis*-RA (Charakida *et al.*, 2004), while patient age and severity of acne appear to be additional factors affecting relapse of skin condition (Chivot *et al.*, 1990).

Following oral administration, three metabolites have been detected in human plasma including 4-*oxo*-isotretinoin (via the oxidation of 13-*cis*-RA), ATRA (through isomerisation of 13-*cis*-RA) and 4-*oxo*-retinoic acid (Accutane: Roche Product information, 1998). The elimination half life of Roaccutane and its metabolites were found to be ~20 hours after a single 80mg dose of Roaccutane that represents ~1mg/kg dose for an adult patient (Bremner, 2003).

1.3.2.2. Side-effects of 13-cis-RA treatment

Since the introduction of Roaccutane onto the market, patients have reported a wide-range of side-effects associated with drug treatment. Roaccutane was found to be highly teratogenic in humans, with exposure anytime after 15-40 days postconception leading to foetal malformations in 25-30% of all cases (Dai *et al.*, 1992). However, a number of risk management programmes have been implemented that aim to prevent the prescription of Roaccutane to pregnant women (Abroms *et al.*, 2006). Several side-effects of Roaccutane are mucocutaneous in nature due to the effects on sebum production, including cheilitis, nasal dryness, dermatitis, skin fragility, acne flare and nail/hair changes (reviewed in (Charakida *et al.*, 2004)). Cheilitis is particularly problematic given that it affects over 90% of all patients and often occurs within the first week of treatment (Ellis *et al.*, 2001). Musculoskeletal problems have also been recorded, with as many as 15% of all patients suffering with myalgias (Fiallo *et al.*, 1996) and 10% displaying diffuse interstitial skeletal hyperosteosis symptoms (DiGiovanna, 2001). Other side effects include ophthalmological problems ranging from dry eyes to corneal opacities, gastrointestinal intolerance in 20% of patients (Bigby *et al.*, 1988) and neurological effects such as headaches in 16% of patients, fatigue, insomnia and others (reviewed in (Hanson *et al.*, 2001)). Perhaps the most controversial and contentious side effect has been Roaccutane's ability to induce adverse psychiatric events including depression, psychosis, suicide ideation and completed suicide (Hull *et al.*, 2005; Hull *et al.*, 2003; O'Donnell, 2003; Strahan *et al.*, 2006).

1.3.3. Clinical evidence of retinoid-induced depression

The evidence supporting retinoid-induced depression comes from a variety of lines of investigation such as cases of hypervitaminosis A, case reports, case series, retrospective studies, government databases and preclinical studies (reviewed in (Bremner *et al.,* 2007)).

1.3.3.1 Hypervitaminosis A and psychiatric effects

A number of studies have highlighted the psychiatric consequences of excess vitamin A consumption. Hypervitaminosis A was first recorded following the consumption of vitamin A-rich polar bear liver and other internal organs by arctic explorers with symptoms of headache, vertigo, drowsiness and irritability (Kane, 1856) and was later reported as *pibloktoq* syndrome found amongst arctic peoples (Landy, 1985). Interestingly, these symptoms would manifest within hours of consumption demonstrating a temporal association and would return upon repeated consumption that is indicative of a challenge/re-challenge effect (O'Connell *et al.*, 2003).

Likewise, some early case reports have shown toxic psychosis following vitamin A treatment for acne (Restak, 1972). In this case, the patient had consumed 50,000 IU of retinol supplements two to three times per day, experiencing depression, elation and insomnia after 6 months, weight loss, blurred vision and agitation after 12 months and finally symptoms of pseudotumour cerebri. All symptoms resolved rapidly after cessation of retinol that is indicative of a de-challenge effect. Another case report documents a patient who consumed 12 times the normal amount of retinol supplements (25,000 IU/day/2 years) and developed depression and poor concentration (McCance-Katz *et al.*, 1992). The symptoms were ablated following cessation of the supplements for two months. Meanwhile, early clinical trials with retinoic acid (100mg-200mg) caused psychological changes in 3 from 30 patients (Stuttgen, 1975).

Other therapeutic retinoids, such as etretinate and acitretin, have been linked with adverse psychiatric events and further suggest a retinoid/depression class effect. Eterinate, a retinoid used for psoriasis,

has been reported to induce depression in three patients and symptoms were resolved following drug withdrawal (challenge/de-challenge effect) and recurred on re-challenge in one patient (Henderson *et al.*, 1989). Acitretin, a metabolite of etretinate, is also used for the treatment of psoriasis and one case report has found treatment induced depression and intense suicidal thoughts (Arican *et al.*, 2006).

1.3.3.2 Case reports/series, government databases & retrospective studies

Since the introduction of Roaccutane in 1982, a consistent number of reports have emerged of the increased incidence of adverse psychiatric events amongst patients that range from depression to completed suicide. Some of the earliest reports consisted of case reports and case series that inferred a causal link between drug administration and adverse psychiatric events in either individuals or medium sized groups (50-100), respectively (summarised in Table 1.2 and reviewed in (Hull *et al.*, 2003; Marqueling *et al.*, 2007; Ng *et al.*, 2003; Strahan *et al.*, 2006)).

The first observation of psychiatric phenomena in 13-*cis*-RA treated patients came in April 1982 soon after the drug was licensed (Meyskens, 1982) and was noted in two patients receiving 3mg/kg/day doses, although it may have occurred in 18 additional patients. In these patients positive de-challenge was recorded, whereby symptoms improved when drug treatment was discontinued. A year following the introduction of the drug, a report showed that 6 out of 110 patients with acne or keratinizing disorders expressed forgetfulness and depressive-like symptoms after receiving 1-2mg/kg/day of Roaccutane (Hazen *et al.*, 1983). Subsequent studies revealed 22 from 94 patients had 'minor' depression, with typical onset occurring later than cutaneous side-effects and generally after one month of treatment (Bruno *et al.*, 1984). Another case series demonstrated a lower incidence of depression, occurring in 1% of patients (7 from 700 patients receiving 0.7mg/kg/day) and symptoms resolved upon de-discontinuation of treatment, with one case of positive re-challenge (Scheinman *et al.*, 1990).

Reference	No. of cases	Adverse psychiatric event	Suicide attempts and completion
Case reports			
Burket *et al*., 1987	1/1	Depressive mood	0/1
Villalobos *et al*., 1989	1/1	Psychosis	0/1
Gatti *et al*., 1991	1/1	Depression	1/1
Cotterill *et al*., 1997	1/1	Depression	1/1
Cott *et al*., 1999	1/1	Bipolar depression	0/1
Middelkoop, 1999	1/1	Depression	1/1
Ng *et al*., 2001	1/1	Depression	1/1
Meyskens, 1982	2/2	Psychological changes	0/2
Duke *et al*., 1993	2/2	Depression	2/2
Bravard *et al*., 1993	3/3	Depression	2/3
Byrne *et al*., 1998	3/3	Depression	2/3
Case series			
Hazen *et al*., 1983	6/110	Depression	0/110
Bruno *et al*., 1984	22/94	Minor depression	0/94
Scheinman *et al*., 1990	7/700	Depression	1/700
ADERS			
Australian ADERS (Adverse Drug Reactions Advisory Committee, 1998)	12	Depression	2
Irish Medicines Board, 1998	6	Unspecified psychiatric events	1
UK Medicine Control Agency (Accutane/Roaccutane Action Group)	236	Suspected psychiatric events	25
Wysowski *et al*., 2001	431	Depression	37
Wooltorton, 2003	56	Depression/suicide ideation	Not recorded
Isotretinoin report MHRA UK, 2004	216	Serious/non-serious depression	55
Prospective survey			
Hull *et al*., 2000	5/121	Depression	0/121
Retrospective case–control study			
Jick *et al*., 2000	1,861/7,535	Depression or psychosis	39/7,535

Table 1.2: Summary of adverse psychiatric event reports associated with 13-*cis*-RA.The table is divided into case reports/series, adverse drug event reporting systems (ADERS), prospective surveys and retrospective case-control studies. The total number of adverse psychiatric events is given from the total number of people observed (if known and not applicable for ADERs), in addition to the description of the adverse psychiatric event measured, with attempted and completed suicide numbers. Modified from Strahan *et al*., 2006.

The adverse effects of Roaccutane have also been monitored using adverse drug event reporting systems (ADERS), which are government-maintained databases that receive voluntary reports from healthcare providers. From the British ADERS and FDA alone, 673 cases of depression were recorded between 1998 and 2003 (Accutane/Roaccutane Action Group; Irish Medicines Board, 1998; Wysowski *et al.*, 2001). The largest study comes from Wysowski who analysed the Adverse Event Reporting System of the FDA for the first 18 years of Roaccutane use and found 431 patients displayed depressive symptoms including 37 who committed suicide and 110 who were hospitalised for depression, suicide ideation or suicide attempt. Meanwhile, the Australian and Canadian ADERSs have reported 12 and 56 cases of depression respectively (Adverse Drug Reactions Advisory Committee, 1998; Wooltorton, 2003).

The examination of 13-*cis*-RA prescription trends from 1982 to 2000 revealed that the largest age group treated was older adolescents (15-19 years) (Wysowski *et al.*, 2002), so it is perhaps unsurprising that three case series have reported adverse psychiatric events in adolescents (Bravard *et al.*, 1993; Byrne *et al.*, 1998; Duke *et al.*, 1993). The symptoms exhibited by adolescents included irritability, sleep disturbances, depression and suicide, with the resolution of suicide ideation in three patients following antidepressant treatment. Meanwhile, a study conducted by the Medicines and Healthcare Products Regulatory Agency (MHRA) in the UK found 216 cases of serious and non-serious depression amongst 13-17 year old Roaccutane patients in addition to 28 cases of suicide ideation and 27 cases of completed suicide (Isotretinoin report MHRA UK, 2004).

In fact, by 1998 the FDA had received such a great number of adverse event reports, that it prompted a change in the Roaccutane product labelling: it now reads ‘Psychiatric Disorders: Accutane may cause depression, psychosis, and rarely, suicidal ideation, suicide attempts and suicide. Discontinuation of isotretinoin therapy may be insufficient; further evaluation may be necessary’(Bull, 2000). Shortly afterwards, the first prospective survey of 124 patients taking Roaccutane (1mg/kg/day for 5 years) was published and found persistent symptoms of depression in 4% of the group (Hull *et al.*, 2000).

Meanwhile, a retrospective case-control study, using Saskatchewan and UK health databases, looked at 7,195 isotretinoin users compared with 13,700 control patients taking oral antibiotics for acne. There were a total of 1,861 patients with psychosis and depression in the isotretinoin group, but there was no significant difference between isotretinoin and control groups (Jick *et al.*, 2000). To date, this study represents the largest cohort of people assessed for an association between 13-*cis*-RA administration and depression, yet no evidence was found for the depressive effects of 13-*cis*-RA. However, there remain a number of methodological issues surrounding this study including a) the Saskatchewan group were diagnosed for depression and psychotic disorders using different diagnostic criteria compared to the UK group, b) different criteria were employed for the diagnosis of suicide in the Saskatchewan group compared with the UK group, c) depression was underestimated by not including other diagnostic codes for depression (in both population groups) and d) the lack of a non-treated control group. The result is that there was the potential for the misclassification of psychiatric disorders along with low agreement for the milder psychiatric symptoms observed that together suggest the pro-depressive effects of 13-*cis*-RA still requires further clarification in human studies.

A recent clinical study has compared Roaccutane patients with an antibiotic control group and sought to investigate possible differences in brain function. Patients underwent PET, with [(18)F]fluorodeoxyglucose, before and after 4 months of treatment with Roaccutane (n=13, 1mg/kg). Roaccutane patients were observed to have decreased brain metabolism in the orbitofrontal cortex, a region associated with dopaminergic pathways and depression pathology, compared with controls (21% reduction compared with a 2% increase in antibiotic controls). However, the severity of depression in the Roaccutane patients as measured by the Hamilton Depression Rating Scale did not appear to alter throughout treatment (Bremner *et al.*, 2005).

Despite the steady number of reports of adverse psychiatric events associated with 13-*cis*-RA use, it should be noted that there exists an opposing viewpoint that 13-*cis*-RA treatment may actually improve psychiatric symptoms (reviewed in (Hull *et al.*, 2003)). Acne itself is known to have a profound effect on psychosocial aspects of patient's lives such as problems with self-esteem, self-

confidence, body image and social withdrawal (Koo *et al.*, 1991; Motley *et al.*, 1989; Shuster *et al.*, 1978). In one study, the dysmorphophobia (the intense and irrational dislike of a part of an individual's own body) exhibited by acne patients was improved by 16 weeks of 13-*cis*-RA treatment (Hull *et al.*, 1991). Meanwhile, a psychiatric assessment of 72 patients before and after receiving 13-*cis*-RA found evidence of 'psychological distress' before treatment, whereas anxious and depressive symptoms appeared to mildly improve post-treatment (Rubinow *et al.*, 1987). Furthermore, a study noted improvements in the emotional state of patients following acne treatment, whom had previously reported feeling 'embarrassment' and 'shame' (Kellett *et al.*, 1999) and another had observed improvements in psychosocial disability (Layton *et al.*, 1997). Many of these studies are limited by a) the assessment of clinical depression via self-report measures rather than by a clinician, b) the multiple comparisons made using different self-report scales and c) the likely measurement of improved patient satisfaction as opposed to actual improvements in clinical depression. Therefore the literature does not provide strong evidence of an improvement in psychiatric symptoms such as depression, but perhaps an improvement in self-image and patient satisfaction (Bremner, 2003).

With the limited data available there is a lack of consensus over the link between Roaccutane treatment and depression-like symptoms. Estimates of incidence range from 1% (Scheinman *et al.*, 1990) to 6% (Hazen *et al.*, 1983), whereas others have found no relationship at all (Jick *et al.*, 2000). The high background incidence of depression and suicide, especially in the adolescent population (Beautrais, 2003; Brent *et al.*, 1999), must be considered and make it difficult to identify small increases related to an additional factor, such as Roaccutane administration. To definitively establish whether a link exists between 13-*cis*-RA therapy and the manifestation of depression in patients, a well-designed double-blind randomized placebo-controlled trial is required, although there are issues with regards to the feasibility and ethicality of such a study. Until such a study is conducted, the use of preclinical data derived from animal models and *in vitro* cell lines will enable the establishment of a biological pathway in which the drug is able to enter the central nervous system and then mediate effects on brain areas related to depression. Such studies will therefore have the role of determining whether an association between 13-*cis*-RA treatment and depression exists.

1.3.4. Preclinical evidence of retinoid-induced depression

Surprisingly little is known about the CNS actions and molecular effects of 13-*cis*-RA due to the small number of preclinical studies published and reinforced by the manufacturers who state the 'exact mechanism of action is unknown'. 13-*Cis*-RA is known to be rapidly isomerised to ATRA *in vitro* using a sebocyte cell-line (but not a keratinocyte cell-line) over the course of 6 hours (Tsukada *et al.*, 2000) and it is therefore believed that 13-*cis*-RA mediates its physiological effects as ATRA. The molecular downstream effects of 13-*cis*-RA are thus either *i)* identical to ATRA or *ii)* similar to ATRA as it is likely to bind to the same repertoire of retinoid receptors (O'Reilly *et al.*, 2008). The implication is that the transcriptional effects of 13-*cis*-RA may be identical to ATRA and therefore like ATRA, 13-*cis*-RA may be capable of inducing neuronal gene transcription and in particular, genes with links to depression such as D2DR and 5-HT_{1A}R.

Behavioural studies have provided putative preclinical evidence of a causal link between retinoids and depression. One study demonstrated that 13-*cis*-RA treatment (1mg/kg/day for 6 weeks) was able to have profound effects on learning tasks and neurogenesis in mice (Crandall *et al.*, 2004). The mice exhibited reductions in cell proliferation in the hippocampus and the subventricular zone, suppression of hippocampal neurogenesis and severe deficits in a spatial learning task called the radial arm maze. Although not directly a measure of depression, the study was able to show molecular and behaviour alterations in a process that has close links with depression pathology (refer to Chapter 1.3.1.6.). However, it is also worth noting that 13-*cis*-RA treatment (7.5mg/kg/day and 30mg/kg/day for 19 weeks) had no effect on spatial learning and memory as measured by the Morris water maze and 8-arm radial maze in adult rats (Ferguson *et al.*, 2007a).

Previous work undertaken in our laboratory group sought to measure the depressive effects of 13-*cis*-RA more directly in juvenile mice (O'Reilly *et al.*, 2006). The study assessed the behavioural effects of 6 weeks of daily 13-*cis*-RA treatment using the FST and TST, which are both validated models of antidepressant efficacy and depression-related behaviour (Cryan *et al.*, 2002). Both tests found

chronic 13-*cis*-RA treatment in juvenile mice was sufficient to create a pro-depressive behavioural profile, with a 58.3% increase in immobility times (in the TST). However, an earlier study that had used the FST and sucrose intake as a measure of anhedonia was unable to show the manifestation of pro-depressive behaviour in adult rats (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b).

1.4. Hypothesis and aims of thesis

The hypothesis of this thesis is that 13-*cis*-RA treatment regulates monoaminergic molecular components, particularly those involved in serotonergic pathways, via RAR-mediated gene transcription and this represents the underlying mechanism that causes pro-depressive behaviour. The aims of my thesis were to test this hypothesis and to further the current understanding of retinoid signalling pathways in the adult brain.

The first aim of my thesis was to establish whether 13-*cis*-RA treatment could induce pro-depressive behaviour in a variety of behavioural models and to dissect the reasons behind the large discrepancy between our previously reported findings (O'Reilly *et al.*, 2006) and that of other studies (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b). We have shown that young mice treated with 13-*cis*-RA (1mg/kg, i.p., daily) for 6 weeks display depression-related behaviour in the FST and TST (O'Reilly *et al.*, 2006), whereas other studies have shown that 10 weeks and 26 weeks of 13-*cis*-RA treatment (7.5,22.5 and 30mg/kg, oral gavage, daily) in adult rats has no behavioural effects in the FST and sucrose consumption test (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b). The methodologies of each respective study differ with respect to the animal model used, the species and age of animals tested and lastly, the dose, treatment length and route of administration of 13-*cis*-RA used.

To assess whether different animal models of depression-related behaviour may contribute to the contrasting findings, I have tested adult rats treated with 13-*cis*-RA (1mg/kg i.p. daily, 2 weeks) in an array of behavioural models including the FST, sucrose consumption test and for the first time in the field of retinoid research, the resident-intruder paradigm coupled with ethological analysis. The

resident-intruder paradigm is able to predict whether drug treatment induces behavioural changes consistent with antidepressant action and enables us to create a more detailed behavioural profile of animals undergoing 13-*cis*-RA treatment. To ascertain the role of the species of animal studied, I have used 13-*cis*-RA-treated juvenile rats in the FST that can be compared with juvenile mice from our previous study (O'Reilly *et al.*, 2006), since the behavioural model and 13-*cis*-RA treatment regime used were identical. To address the importance of the age of the animal tested, I have used both juvenile (4 weeks old at start of treatment) and adult (8 weeks old at start of treatment) rats in the FST and sucrose consumption test. It may be that juvenile rats, because of their developmental stage, are particularly vulnerable to the effects of retinoid signalling compared with adult rats (see Chapter 3).

The second aim was to determine whether monoaminergic gene expression can be altered by 13-*cis*-RA treatment in brain regions associated with depression pathology, such as the hippocampus and raphe nuclei. Previous studies have indicated that 5-HT_{1A}R (Charest *et al.*, 1993) and numerous other neuronal genes are regulated by retinoids (reviewed in (Lane *et al.*, 2005)), while there is converging evidence for retinoid receptor expression in the mouse hippocampus, although expression had not been similarly tested in the raphe nuclei (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). I have therefore sought to establish the expression profile of retinoid receptors (RARs and RXRs) in the rat raphe nuclei tissue, in parallel with an *in vitro* rat raphe RN46A-B14 cell line and the rat hippocampus tissue. Upon the successful expression of retinoid receptors in the tissue/cell line, I sought to measure the gene expression of monoaminergic components such as the 5-HT_{1A}R, 5-HT_{1B}R, TPH2, MAOA, D2DR and COMT enzyme, which may be regulated by retinoids. In this thesis, I have also expanded upon previously published work from our group that demonstrates the ability of 13-*cis*-RA to increase 5-HT_{1A}R and SERT protein levels *in vitro* using the serotonergic RN46A-B14 rat cell line (O'Reilly *et al.*, 2007), by determining whether 13-*cis*-RA treatment can alter monoaminergic protein expression *in vivo* using rat brain raphe nuclei and hippocampal tissue (see Chapter 4).

Finally, if monoaminergic gene expression is compromised then neurotransmitter levels could be altered which would contribute to the increased incidence of depression observed in 13-*cis*-RA

patients. Therefore my final aim was to determine the effect of 13-*cis*-RA treatment on neurotransmitter levels including NA, DA and 5-HT, along with the serotonin metabolite 5-HIAA, in the prefrontal cortex, hippocampus and raphe nuclei tissue of chronically treated rats, as well as the levels of 5-HT and 5-HIAA in plasma (see Chapter 5).

CHAPTER 2

General Methods

2.1. Introduction

We designed and implemented two experimental approaches to examine retinoid-induced depression. The first approach was an *in vivo* study that allowed us to research the behavioural effects of 13-*cis*-RA treatment for two and six weeks in both juvenile and adult rats. The second approach involved treating a cell line of rat raphe derived neurons (RN46A-B14 cells) with 13-*cis*-RA. Changes in gene expression and protein levels of depression-related monoaminergic components were analysed in rat tissues and cell lines, following cessation of 13-*cis*-RA treatment. The experimental techniques employed are described below.

2.2. *In vivo* studies: rats

All experiments were carried out under a project licence held under the Animals (Scientific Procedures) Act 1986 and in accordance with the UK Home Office guidelines. In all behavioural experiments, age-matched male Wistar rats (Charles River, UK) were used. The putative age-related effects of 13-*cis*-RA treatment (discussed in Chapter 3) were determined using juvenile (4 weeks old) and adult rats (8 weeks old) at the start of 13-*cis*-RA treatment respectively. Previous work has shown that rats between postnatal 28 and 42 exhibit juvenile-typical neurobehavioural characteristics and can therefore be regarded as juveniles (Spear, 2000; Spear *et al.*, 1983). Rats of 8 weeks of age are generally regarded as adults as even the most broad estimate of the adolescence is between weaning and postnatal day 60 (Spear, 2000). All animals were group housed (n=3 or 4) in cages containing sawdust bedding with no environmental enrichment. Animals were usually maintained under daylight conditions (12 h on/ 12 h off, lights on at 07:00 h), while food and water were provided *ad libitum*. Upon arrival, rats were weighed and subsequently measured on a weekly cycle throughout the course of the experiments. Consistent weight gain was used as an indicator of good general health and level of stress caused by handling or intraperitoneal injections.

Control tissues (required for primer validation and gene of interest expression profile experiments) were obtained from adult Wistar rats (University of Bath, 250g-350g) and brain regions were microdissected as per Chapter 2.2.2, followed by RNA isolation as per Chapter 4.2.2.

2.2.1. 13-*Cis*-RA treatment regime

All animals received daily intraperitoneal injections at a volume of 1ml/kg body weight. Similar to previous animal studies looking at the chronic effects of 13-*cis*-RA treatment (O'Reilly *et al.*, 2006), rats received treatment for 6 weeks with the exception of the resident-intruder paradigm (two week treatment, see Chapter 3). Vehicle control groups received sterile saline solution (0.9% w/v sodium chloride) with dimethyl sulphoxide (DMSO, Eur Ph, ICMD UK Ltd) at a ratio of 1:1 v/v. Drug treated groups received 1mg/kg 13-*cis*-RA (Sigma-Aldrich, UK) dissolved in 1:1 v/v DMSO:saline. This dose of 13-*cis*-RA is in the range of doses widely used to treat acne in patients (0.5 to 2 mg/kg/day) and we have previously achieved plasma levels of 1.51 ± 0.05µg/ml in animals that is comparable to plasma levels in patients (Kerr *et al.*, 1982; O'Reilly *et al.*, 2006). Stock solutions of 2mg/ml 13-*cis*-RA were prepared in DMSO and frozen at -20°C. When required, an equal volume of sterile saline was added to the stock solution and allowed to reach room temperature before injection. All 13-*cis*-RA preparation took place under red light because of its photosensitivity and potential to degrade in normal light (O'Reilly *et al.*, 2006). In all experiments, rats received daily intraperitoneal injections on alternating sides of the peritoneal midline to reduce irritation, at 16:00-17:00 h to avoid any acute effect of the injections on behavioural testing.

2.2.2. Microdissection of brain regions

After cessation of retinoid treatment, both 13-*cis*-RA-treated and vehicle-treated rats were killed by cervical dislocation with subsequent decapitation. The whole brain was removed rapidly and kept on dry ice. The microdissection of the prefrontal cortex was performed by making a coronal cut (freehand with a razor blade, with the aid of a rat brain atlas (Paxinos *et al.*, 1998)) of the anterior portion of the brain and the olfactory bulb was removed. This was followed by cutting coronal slices

anterior of the cerebellum and subsequently dissecting a triangular region below the periaqueductal gray of each slice, containing the raphe nuclei. The remaining section of brain was cut in the sagittal plane and the hippocampus was removed from each hemisphere. All microdissection procedures were performed on ice. For each microdissected brain region, RNA isolation, protein isolation or high – performance liquid chromatography (HPLC) analysis was performed (see Chapter 4.2.2., Chapter 4.2.6.1. and Chapter 5.2.1., respectively). All samples were stored at -80°C.

2.2.3. Retinoid extraction

Plasma levels of retinoids were determined for all animals who had completed 6 weeks of either 13-*cis*-RA or vehicle treatment. Firstly, trunk blood was collected and immediately placed on ice for 30 min to allow for coagulation. Blood was then centrifuged at 1000g for 20 min and the platelet-poor plasma (PPP) supernatant was collected. To extract retinoids from plasma, 210µl of acetonitrile/butanol solution (1:1 v/v, with 5mg of butylated hydroxtoluene) was added to 300µl of plasma sample and vortexed for 45 s. This was followed by the addition of 180µl of fresh saturated K_2HPO_4 and subsequently vortexed for 10 s. The samples were centrifuged for 10 min at 4°C and the upper phase was collected, followed by a further centrifugation step for 5 min. The clear yellow upper phase of plasma was collected (Lane *et al.*, 1999; Liu *et al.*, 2005).

Additionally, 13-*cis*-RA was added to plasma samples deriving from rats that were not treated with either vehicle or 13-*cis*-RA, thereby creating plasma samples with known retinoid concentrations ranging from 0.0003µg/µl to 0.03µg/µl (made from a stock solution of 0.003mg/µl 13-*cis*-RA in 100% ethanol). Retinoids in these samples were extracted as previously described. All plasma samples (150µl, treated and untreated plasma samples) were loaded into a quartz cuvette and the absorbance was measured spectrophotometerically at 354nm. The absorbance of plasma samples of known retinoid concentration allowed for the construction of a standard curve with absorbance at 354nm plotted against retinoid concentration in µg/µl. Subsequent absorbance measurements of plasma samples of unknown retinoid concentration (from vehicle and 13-*cis*-RA-treated rats) were

plotted on the standard curve and retinoid concentrations were derived. All procedures were performed in red light given the light sensitivity of 13-*cis*-RA.

2.3. *In vitro* studies: Cell lines

The RN46A-V1 (or RN46A) cell line is derived directly from embryonic day 13 rat medullary raphe cells infected with a retrovirus encoding the temperature-sensitive mutant of SV40 large T antigen (White *et al.*, 1994; White *et al.*, 1992). The RN46A cell line is neuronally restricted, with a fibroblast-like morphology, at permissive temperature (33°C) and constitutively differentiates towards a bipolar neuronal-like morphology following a shift to nonpermissive temperature (39°C). Long-term treatment with BDNF enhances differentiation towards a serotonergic-like phenotype as demonstrated by 5-HT synthesis and release (White *et al.*, 1994) and 5-HT_{1A} autoreceptor binding (Eaton *et al.*, 1995). Recently, RN46A cells treated with BDNF were shown to have high cell body 5-HT immunoreactivity and a high expression of 5-HT_{1A} and 5-HT_{1B} receptors (Rumajogee *et al.*, 2006). Furthermore, a subclone of RN46A cells has been stably transfected with BDNF, known as RN46A-B14 cells (Eaton *et al.*, 1996). The RN46A-B14 cells were shown to secrete BDNF and synthesize more 5-HT than the RN46A parent cell line.

Culture conditions for RN46A-B14 cells (kind gift of Scott R. Whittemore) were as described by White *et al.*, 1994 and Eaton *et al.*, 1995. RN46A-B14 cells were grown in sterile filtered neurobasal medium (CNS medium), with 10% foetal bovine serum (FBS), 0.5mM glutamine and 1% penicillin/streptomycin at 33ºC. Once 60% confluent, they were trypsinised (trypsin) and resuspended in differentiation medium made from sterile filtered Dulbecco Modified Eagle's minimum essential media (DMEM)/F12 with 1% FBS, 1µg/ml bovine transferrin, 5µg/ml insulin, 100nM putrescine 20nM progesterone and 1% penicillin/streptomycin (Eaton *et al.*, 1996; Rumajogee *et al.*, 2006). All RN46A-B14 cells were subsequently plated into each well of a 6-well plate and incubated at 39ºC (Day 0). On day 2 and day 6 of differentiation, 25ng/ml of BDNF (Pepro Tech) was

added to all wells (Rumajogee *et al.*, 2006) to increase the serotonergic properties of the RN46A-B14 cells.

The MDA-MB-468 cell line (ATCC LGC Promotech, Middlesex, UK) derives from human breast adenocarcinoma cells isolated from a 51-year-old black female and was used as a non-neuronal negative control for Western blotting. Cells were grown at 37°C in DMEM, supplemented with 4mM L-glutamine, 10% foetal calf serum, 2.5µg/ml fungizone (amphotericin B) and 50µg/ml penicillin/streptomycin. All culture media were obtained from Invitrogen (UK) or Sigma (UK) unless otherwise stated.

2.3.1. RN46A-B14 retinoid treatment

After 6 days of RN46-B14 cell differentiation, cells were treated with ethanol (0.5% final culture concentration), 2.5µM 13-*cis*-RA or 10µM of 13-*cis* RA (13-*cis*-RA dissolved in 100% ethanol). The concentrations of 13-*cis*-RA were chosen based on reports that the maximum steady-state plasma concentration of 13-*cis*-RA reaches 731.98 ± 361.86 ng/ml (2.5 µM) and the 10µM concentration may be reached soon after 13-*cis*-RA administration (O'Reilly *et al.*, 2007). 13-*Cis*-RA was added in low level light and all treatments were applied to two wells of the 6-well plate. The 6-well plate was subsequently incubated in the dark for 48 h at 39ºC. At the end of the experiment, the RNA/protein of RN46A-B14 cells were isolated as described in Chapter 4.2.2. and Chapter 4.2.6.1., respectively.

Chapter 3

The behavioural effects of 13-*cis*-RA administration in adult and juvenile rats

3.1 Introduction

Given the controversial findings regarding the use of 13-*cis*-RA and depression in humans, there has been an interest in utilising animal studies. Human studies have two major confounding factors: severe acne can itself have significant psychological and emotional impact that may induce or increase susceptibility to depression (Fried *et al.*, 2006; Gupta *et al.*, 1998; Kellett *et al.*, 1999) and secondly, human studies are often incomplete with the length of treatment, prior psychiatric history, dosage used and follow-up assessments often omitted or not recorded (Strahan *et al.*, 2006). The use of animal models to study depression-related behaviour also presents a number of problems, given that some endophenotypes such as suicidal ideation do not occur in animals, interindividual and interspecies variabilities in behavioural responses to the test situation and the validity of animal models to reflect human emotions (Cryan *et al.*, 2005a). Although animal models can never be completely congruent with the human disorder, there are minimal criteria for the animal model to be valid (Nemeroff, 2002). Many animal models have face validity which refers to the similarity between the behaviour exhibited in the animal model and the human disorder, others may have high predictive validity whereby changes in the human subject can be predicted from changes in the animal model and finally other models demonstrate the ability to reflect the pathology of the disorder/disease and are said to have high construct validity (Geyer *et al.*, 1995).

There are a number of well-established, pharmacologically validated paradigms for investigating depression-related behaviours (Cryan *et al.*, 2002). Stress and trauma are thought to be factors that predispose humans to depression and therefore depression can be perceived to be an inability to cope with stress. This enables the construction of animal models of depression-related behaviour that are based on social stress such as the resident- intruder paradigm or environmental stress such as the chronic mild stress paradigm (Anisman *et al.*, 1990). Social stress models include social hierarchal paradigms whereby submissive rats become more aggressive through repeated antidepressant treatment (Mitchell *et al.*, 1992b) and the resident-intruder paradigm where the aggression shown in

social interactions of the resident rat towards the intruder is varied by antidepressants (Mitchell, 2005).

The resident-intruder paradigm provides an ethologically relevant animal model by which the effects of acute and chronic antidepressant treatment (including electroconvulsive shock) on rodent non-social, social and agonistic (i.e. aggression and flight) behaviours may be examined (Mitchell, 2005; Mitchell *et al.*, 2003; Mitchell *et al.*, 1992b). The ability of chronic antidepressant treatment to increase rodent aggressive behaviour is indicative of increased assertive behaviour and mirrors changes in human behaviour observed during recovery from depressive illness (Bond, 2005; Dixon *et al.*, 1989; Eisen, 1989; Khan *et al.*, 1989; Willner *et al.*, 2002). In contrast, acute treatment with antidepressant drugs selectively reduces rodent aggression/assertiveness and may therefore predict increased depressive symptomatology, including suicide ideation, suicide attempts and self-harm (Bond, 2005; Mitchell, 2005; Möller *et al.*, 2008). Thus the resident-intruder paradigm, coupled with ethological analysis, has the ability to predict whether drug treatment may induce behavioural changes consistent with either an antidepressant or a pro-depressant action.

Alternative paradigms for measuring depression-like behaviour rely on environmental stress as opposed to social stress (Maier, 1984). The premise of the learned helplessness paradigm is that repeated exposure to uncontrollable electric shocks can induce escape-related deficits in animal that can be reversed by antidepressants (Vollmayr *et al.*, 2001). A major concern with this paradigm is that depression-like behaviour persists for only 2-3 days after cessation of shocks (Weiss *et al.*, 1998). The effect of shocks can be prolonged by incorporating aspects of mild repeated, unpredictable and uncontrollable stimuli such as restraint and novel housing, known as the chronic mild stress paradigm (Gambarana *et al.*, 2001). The chronic mild stress paradigm and the learned helplessness paradigm both induce escape-related deficits and also anhedonia (Naranjo *et al.*, 2001), which is a common endophenotype of depression characterised by reduced reward sensitivity. Following chronic mild stress, anhedonia has been measured as a significant decrease in the consumption of palatable sucrose solutions (Papp *et al.*, 1991); a process that can be reversed by antidepressants (Muscat *et al.*, 1992b).

Interestingly, DA agonist treatment appears to reverse anhedonic effects which suggests that the model is closely linked to the dopaminergic pathways involved in depression (Muscat *et al.*, 1992a).

The FST (Porsolt *et al.*, 1977) is a variation of the learned helplessness model and has been developed for use with both rats and mice (Porsolt, 2000). The premise of the model is that following escape-oriented movements, the rat or mouse adopts an immobile posture when placed in an escapable container of water. The immobility observed is likely to be reflecting a deficit in escape-related behaviour also known as behavioural despair or the development of passive behaviour as a result of an inability to cope with stressful situations (Lucki, 1997). The TST is a related model, whereby mice are suspended by their tail and exhibit passive immobility following escape-related behaviour (Steru *et al.*, 1985).

While the original FST was able to measure the effects of acute treatment with TCAs, MAOIs and electroconvulsive shock via a reduction in immobility times of rats and mice (Borsini *et al.*, 1988), it was unreliable in measuring the acute effects of SSRIs (Borsini, 1995). This led to the modification of the FST, with the introduction of a pre-swim session (Detke *et al.*, 1995; Lucki, 1997), that enabled the effects of fluoxetine, paroxetine and sertraline to be measured as reductions in immobility times (Detke *et al.*, 1995). Moreover, the modified FST showed that SSRIs such as fluoxetine increased swimming behaviour whereas NA reuptake inhibitors such as desipramine and reboxetine increased climbing behaviour (Cryan *et al.*, 2005b). It has been suggested that the FST is simply a reliable model of antidepressant efficacy (Gardier *et al.*, 2001), however pro-depressive effects such as withdrawal from chronic amphetamine administration (Cryan *et al.*, 2003) and intracerebroventricular injections of urotensin-II (Do-Rego *et al.*, 2005) both increase immobility time in the FST and TST.

Previous studies have utilised behavioural paradigms to elucidate the chronic effects of 13-*cis*-RA treatment. Results from our laboratory have shown increased immobility times in the FST and TST following chronic treatment of juvenile mice with 13-*cis*-RA (1mg/kg/day/ip for 6 wks) (O'Reilly *et al.*, 2006); results consistent with an increase in depression-related behaviours. On the other hand,

behavioural studies in adult Wistar rats have shown that chronic treatment (> 7.5 mg/kg/day/gavage for 3-12 wks) with 13-*cis*-RA does not have a pro-depressive effect in the FST or sucrose anhedonia paradigm (Ferguson *et al.,* 2005a; Ferguson *et al.*, 2007b). These studies suggest that the behavioural effects of 13-*cis*-RA may be age specific (adult vs juvenile), species specific (rats vs mice), or sensitive to the different treatment regimes employed, such as the route of administration (oral gavage vs intraperitoneal injection) and dose used (7.5 or 30mg/kg/day vs 1mg/kg/day) (Ferguson *et al.*, 2007a; Ferguson *et al.*, 2005a; O'Reilly *et al.*, 2006).

In this chapter I have tested whether 13-*cis*-RA can induce an increase in depression-related behaviours in adult and juvenile rats following chronic 13-*cis*-RA or vehicle administration. I have tested the behaviour of adult rats in the resident–intruder paradigm and addressed whether, because of their developmental stage, juvenile rats may be particularly vulnerable to the effects of retinoid signalling compared with adult rat behaviour in the FST and sucrose consumption test. Locomotor behaviours were also examined to control for any confounding effects of 13-*cis*-RA on locomotion since such behaviours have been reported to be influenced by retinoids (Krezel *et al.*, 1998). The blood plasma levels of retinoids from treated animals were analysed via retinoid extraction methods to confirm the 13-*cis*-RA treatment regime was sufficient and consistent with previously reported studies.

To test the involvement of the 5-HT system and specifically 5-HT_{1A}R s in the pro-depressant actions of 13-*cis*-RA I have used the 8-OH-DPAT-induced hypothermia paradigm. The pharmacological activation of postsynaptic 5-HT_{1A}Rs with selective 5-HT_{1A}R agonist 8-OH-DPAT is known to induce hypothermia in rats (Bill *et al.*, 1991). Here it was used to assess whether 6 weeks of 13-*cis*-RA treatment could alter 5-HT_{1A}R receptor function/number. The degree of hypothermia induced is thought to be related to the level of [^{3}H]8-OH DPAT binding and in turn, the number of 5-HT_{1A}Rs, in some brain areas such as the frontal cortex (Knapp *et al.*, 1998).

3.2 Methods

3.2.1 Animals

In the resident–intruder paradigm, rats at 3-4 wks of age were maintained under reversed daylight conditions (12 h on/12 h off, lights on at 19:00 h) for at least 4 weeks and were 8 weeks old (adult) at the start of the experiment. Rats were group housed (n=4) with food and water provided *ad libitum*. Rats were designated 'resident' (220–340 g pre-treatment weight, n=8 per treatment group) and 'intruder' (230–300 g pre-treatment weight, n=8 per treatment group) and were obtained from different suppliers to ensure that resident animals (Charles River, UK) had never been in contact with intruder animals (University of Bath).

Animals used in the FST, sucrose consumption, open field test and 8-OH-DPAT-induced hypothermia paradigm were maintained under standard daylight conditions (12 h on/12 h off, lights on at 07:00 h). Food and water were provided *ad libitum*, except to those rats undergoing the sucrose consumption test. All animals were treated with either vehicle or 13-*cis*-RA daily for 6 weeks. Juvenile rats were 4 weeks old at start of treatment (n=8 per group, 70-100g, Charles River, UK), whereas adult rats were 8 weeks old (n=8 per group, 270-305g, Charles River, UK) and were housed in groups of 4. Animals used to test 8-OH-DPAT-induced hypothermia were all adults and 8 weeks old at start of treatment (n=12/treatment group, housed in groups of 3, Charles River, UK).

3.2.2. Resident-intruder paradigm

In all resident-intruder studies only the resident rats received drug or vehicle and two groups of resident rats (and associated intruder conspecifics) were studied concurrently. Over the course of 4 weeks, a group of 4 resident rats experienced 4 weekly encounters with 4 different intruder rats, such that each resident rat encountered each of 4 intruder rats (shown in Table 3.1). On the first occasion that each resident rat had a social encounter with an unfamiliar intruder rat, no treatment was given to provide a baseline behavioural profile (denoted as day 0). Following this baseline, resident rats were treated with either vehicle or 13-*cis*-RA daily for 14 days. Social encounters were performed after 7

	Day 0 (pre-treatment)	Day 7	Day 14	Day 21 (post-treatment)
Resident 1	Intruder 1	Intruder 2	Intruder 3	Intruder 4
Resident 2	Intruder 2	Intruder 1	Intruder 4	Intruder 3
Resident 3	Intruder 3	Intruder 4	Intruder 1	Intruder 2
Resident 4	Intruder 4	Intruder 3	Intruder 2	Intruder 1

Table 3.1: Resident-intruder encounters. The experiment was designed such that each resident rat encounters each of the intruders over the 4 weeks.

and 14 days of treatment. The final social encounter was then performed 7 days after the cessation of drug treatment (day 21 or post-treatment).

All social encounters were performed on the test day between 10:00 h and 16:00 h. Prior to each test day resident rats were separated from their group cages and housed individually for 3 days. At the start of each social encounter test, the home cage containing the resident rat was positioned inside the recording cabinet for 30 min to allow for habituation, following which the intruder conspecific was introduced (Mitchell *et al.*, 1992b). The ensuing social encounter was recorded on video tape for 10 min under low-intensity red light (2 lux at the cage floor). At the end of each recording session both resident and intruder rats were returned to their respective group cages. The analysis of resident rat social behaviour, during video playback, involved scoring the occurrence of each of the various behaviours and postures summarised in Table 3.2 (Grant, 1963). The scores for each behaviour/posture were grouped according to their motivational category for each resident rat and the total score for each category expressed as a percentage of the total number of behaviours observed for that animal. All ethological analyses were conducted by Dr. Paul Mitchell and performed blind to the resident rat's treatment group.

Motivational category	Behavioural element
EXPLORATION	Locomotion, rearing
INVESTIGATION	Approach, follow, stretched attention, to-fro, walk round/circle/side, nose and investigate, sniff genitalia, tail rattle
SEXUAL	Mount*, attempt mount, lick penis
AGGRESSION	Aggressive groom, aggressive posture, attack, bite, offensive sideways, offensive upright, pull, threat/thrust
FLIGHT SUBMIT	Defensive sideways, defensive upright, submit
FLIGHT ESCAPE	Attend, crouch, elevated crouch, flag and evade, retreat, under food hopper
MAINTENANCE	Digging, drinking*, eating*, licking, scratching, head/body shake, washing

Table 3.2: Ethogram summarizing the behaviours expressed by rats during social encounters. * indicates that these behaviours were not recorded during the resident-intruder studies because full mating behaviour does not occur between male cohorts and food/water was not provided during the encounter. Adapted from Grant, 1963.

3.2.3. Forced swim test

Adult and juvenile rats were treated daily for a total of 6 weeks with either 1mg/kg 13-*cis*-RA or vehicle (n=8 per treatment group) and were behaviourally tested after 2 and 6 weeks of treatment. Animals tested via the modified FST (Lucki, 1997) underwent a 15 min pre-swim that was followed by a 6 min test session 24 h later. For the swim sessions, rats were placed in a glass beaker (height 44 cm, diameter 22 cm) with water at a height of 34 cm (Detke *et al.*, 1996) and a temperature of 25 °C (±1 °C). Behaviour was recorded for the duration of both the pre-swim and swim test sessions using a camcorder (Sony DCR-SR52). On completion of the swim session, rats were removed from the water, dried and returned to the group home cage. The water was replaced between trials.

The analysis of behaviour during the 6 min swim test session (and first 6 min of the pre-swim test) was conducted blind to treatment. The time spent climbing, swimming and immobility was recorded. Climbing was defined by vertical escape behaviour, swimming was defined by diving and circular paddling around the beaker and immobility taken as the minimal activity required to stay afloat (Cryan *et al.*, 2002).

3.2.4. Sucrose consumption test

Preliminary sucrose consumption tests were conducted following 16 h food and water deprivation in rats, using both 1% (w/v) and 3% (w/v) sucrose solution and measured over a 1 h and 2 h time period. It was found that the 1% (w/v) sucrose concentration was sufficiently hedonic/pleasurable and accurately measured over a 1 h and 2 h time period and therefore used in subsequent sucrose consumption tests (Muscat *et al.*, 1992a). During the test, rats were housed individually and the total amount of sucrose consumed was recorded, corrected for the body weight of each rat and expressed as g sucrose consumed/kg body weight.

3.2.5. Open field test

Given that the FST relies on detecting changes in immobility with concomitant changes in swimming/climbing, it is important to demonstrate that drug treatment does not alter locomotor performance. The locomotor activity of vehicle and 13-*cis*-RA-treated rats undergoing the FST and sucrose consumption tests was assessed in a circular open field divided into 8 segments by equally spaced radii (765 mm diameter×185 mm high) and further divided with an internal circular perimeter (660 mm diameter), shown in Figure 3.1. Rats were placed in the centre of the open field (segment 11, facing towards the centre) and the behaviour was recorded for 10 min under low light conditions (10 lux) using a camcorder (Sony DCRSR52). Analysis of behaviour in the open field was performed blind to treatment and both line crossings and vertical rearing behaviour were scored. A line crossing was defined as when all 4 paws were in one particular segment. Vertical rearing behaviour was defined as a lifting of the two front paws off the ground, but not for grooming.

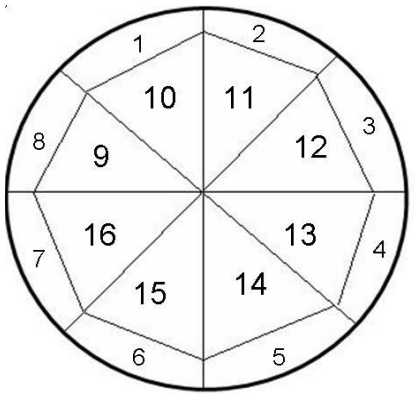

Figure 3.1: Schematic of the open field. All rats were tested in the open field as a control for locomotion behaviour. They were placed in segment 11 facing towards the centre in low light levels. The first 5 s of behaviour in the open field was omitted in the analysis.

The order in which behavioural testing occurred was firstly the sucrose consumption test, followed by open field test and finally the FST such that the most stressful test occurred last. Tests were arranged so that at least 16 h elapsed between test sessions and all behavioural tests were conducted during the light cycle between 09:00 am and 16:00 pm. The treatment of rats continued throughout the duration of behavioural testing and were performed at least 2 h after behavioural testing. Individual rats were tested in a random order in each behavioural test.

3.2.6. 8-OH-DPAT-induced hypothermia

Preliminary experiments were employed in control non-treated rats to confirm an effective dose of 8-OH-DPAT (0.3mg/kg and 0.5mg/kg, s.c.) that produced reliable and measurable hypothermia (>1°C drop over 30 min). Subsequently, a further cohort of adult rats were treated daily with either vehicle (n=12) or 13-*cis*-RA (n=12) for 6 weeks in line with previous cohorts tested behaviourally. Upon completion of treatment, 8-OH-DPAT-induced hypothermic responses were assessed. All rats had a baseline body temperature reading taken (t= -30 min) using a rectal probe (Microprobe Thermometer and rectal probe for rats, Physitemp instruments) and were subsequently measured again to create a further baseline reading (t=0 min). Rats were then injected subcutaneously with either 2.5ml/kg saline

(n=6/vehicle and 13-*cis*-RA-treated rats) or 0.3mg/kg of the 5-HT_{1A}R agonist 8-OH DPAT (n=6/vehicle and 13-*cis*-RA-treated rats, in a volume of 2.5ml/kg saline). Further body temperature measurements were taken at t=15 min, t=30 min and t=60 min. Maximal hypothermic responses were expected to occur at 30 minutes following administration (Bill *et al.*, 1991).

The following day, all rats had a baseline body temperature reading (t= -30 min) followed by the immediate subcutaneous injection of 0.1mg/kg of the 5-HT_{1A}R antagonist WAY-100635 (n=6/vehicle and 13-*cis*-RA-treated rats, in a volume of 1ml/kg saline). After 30 minutes had elapsed, all rats had their body temperatures measured (t=0 min) and then an immediate subcutaneous injection of either 2.5ml/kg of saline (n=6/vehicle and 13-*cis*-RA-treated rats) or 0.3mg/kg of 5-HT_{1A}R agonist 8-OH-DPAT in all rats (n=6/vehicle and 13-*cis*-RA-treated rats, made in 2.5ml/kg of saline). As before, additional temperature measurements were made at t=15 min, t=30 min and t=60 min. All temperature readings were subsequently plotted as temperature changes relative to baseline readings.

3.2.7. Statistical analysis of behavioural studies

For the resident-intruder paradigm studies, the data from the two groups of 4 resident rats were grouped (i.e. n=8 for each treatment group) and the mean ± SEM for both the percentage values of each motivational category, and the total number of behaviours/postures observed, were calculated. All data were subjected to square root transformation prior to statistical analysis. 1-Way ANOVA (with 'treatment' as the dependent measure) with repeated measures over the four test sessions was employed to identify significant differences between the categories of behaviour of the drug and vehicle-treated resident rats. Following identification of time*treatment interactions or main effects of time, within-treatment comparisons were further analysed by post-hoc 1- way ANOVA tests (with 'time' as the dependent measure) following a priori decisions regarding appropriate multiple comparisons.

Where appropriate, pre-treatment levels of behaviour (day 0) were compared to the levels of behaviour observed following 7 and 14 days of treatment and 7 days following the cessation of treatment (day 21). In addition, day 14 data (treatment) were compared to day 21 data (7 days post-treatment). Between-treatment comparisons (following identification of time*treatment interactions or main effects of treatment) were further analysed by post-hoc 1-way ANOVA (with 'treatment' as the dependent measure) between the drug- and vehicle-treated resident rats at each time point for that behavioural category. In all cases, P values arising from repeated comparisons ANOVA are quoted following Huynn–Feldt correction.

Two-way ANOVAs (with ‘treatment’ and ‘age’ as dependent measures) with repeated measures over the two test sessions (week 2 and week 6) were performed on data from the FST, sucrose consumption and open field tests. Appropriate multiple comparisons were made and analysed by post-hoc 1-way ANOVA tests. Hypothermic responses following 8-OH-DPAT treatment were calculated as changes in body temperature from an initial baseline temperature and analysed using one-WAY ANOVAs with repeated measures (body temperature readings were taken at t= -30 min, t= 0 min, t= 15 min, t= 30 min and t= 60 min). Between group comparisons at each time point were made using an unpaired t-test. Differences in body weight and sucrose solution consumed were compared using an unpaired t-test. Values were taken to be significant when $P<0.05$. All values are mean ± SEM unless otherwise stated.

A trend was taken to be a *P* value greater than (and not including) 0.05 and less than 0.15 (i.e. $0.05<P\leq0.15$), following a t-test. This definition of a trend is used throughout the remainder of the thesis.

3.3. Results

3.3.1. The effect of 13-*cis*-RA treatment on weight gain

All rats undergoing behavioural testing were weighed weekly and body weights were expressed as either mean group weight or mean group weight gain as a percentage of each rat's starting weight. In the resident–intruder experiment, the mean body weight of control and 13-*cis*-RA-treated resident rats, one week prior to starting the experiment ("starting weight"), was 269 ± 15 g and 269 ± 9 g, respectively (n=8 per group). As shown in Figure 3.2A, total group weights steadily increased over the course of the experiment, such that mean group weights for vehicle and 13-*cis*-RA-treated were 425.1 ± 8.7g and 410.4 ± 14g, respectively. At all time points there was no significant difference between vehicle and drug- treated groups ($P>0.05$). Additionally there was no significant difference in weight gain during the resident–intruder experiment with weight gain at 21 days being 161 ± 9% and 155 ± 9% for vehicle and 13-*cis*-RA-treated rats, respectively (shown in Figure 3.2B).

Rats undergoing chronic treatment for six weeks, had weight measurements taken one week prior to treatment ("starting weight") and after every subsequent week of treatment. The mean starting body weight of control and 13-*cis*-RA-treated juvenile rats was 91 ± 2.6g and 85 ± 2.9g, respectively (n=8 per group) and were not significantly different. For adult vehicle and 13-*cis*-RA-treated rats there was a significant difference in starting weight (285.7 ± 2.7g and 275.0 ± 1.6 g, respectively, n=8 per group, $P<0.05$). Upon completion of 6 weeks of 13-*cis*-RA treatment, body weights were 418.4 ± 12.0g and 410.9 ± 6.4g for juvenile controls and 13-*cis*-RA-treated rats respectively, while body weights for adult controls and 13-*cis*-RA-treated rats were significantly different at 480.8 ± 9.6g and 449.4 ± 7.1g, respectively ($P<0.05$). However, when measuring weight gain as a percentage of weight gained from the starting weight, 13-*cis*-RA had no effect between vehicle and treated adult (155 ± 4% vs 155 ± 3% respectively, shown in Figure 3.3A) or juvenile (447 ± 10% vs 444 ± 9% respectively, shown in Figure 3.3B) rats undergoing the FST, sucrose consumption and open field tests. There is evidently a larger weight gain in juvenile rats in both control and treated groups, compared to adult animals corresponding to normal growth rates for Wistar rats (Charles River, UK).

The weekly body weights of adult rats required for testing different hypothermic responses were also measured (data not shown). Starting weights of vehicle and 13-*cis*-RA-treated rats were 321.7 ± 4.4g and 322.7 ± 2.5g, respectively (P>0.05, n=12), and by the end of 6 weeks treatment were 493.3 ± 9.4g and 492.8 ± 9.0g, respectively.

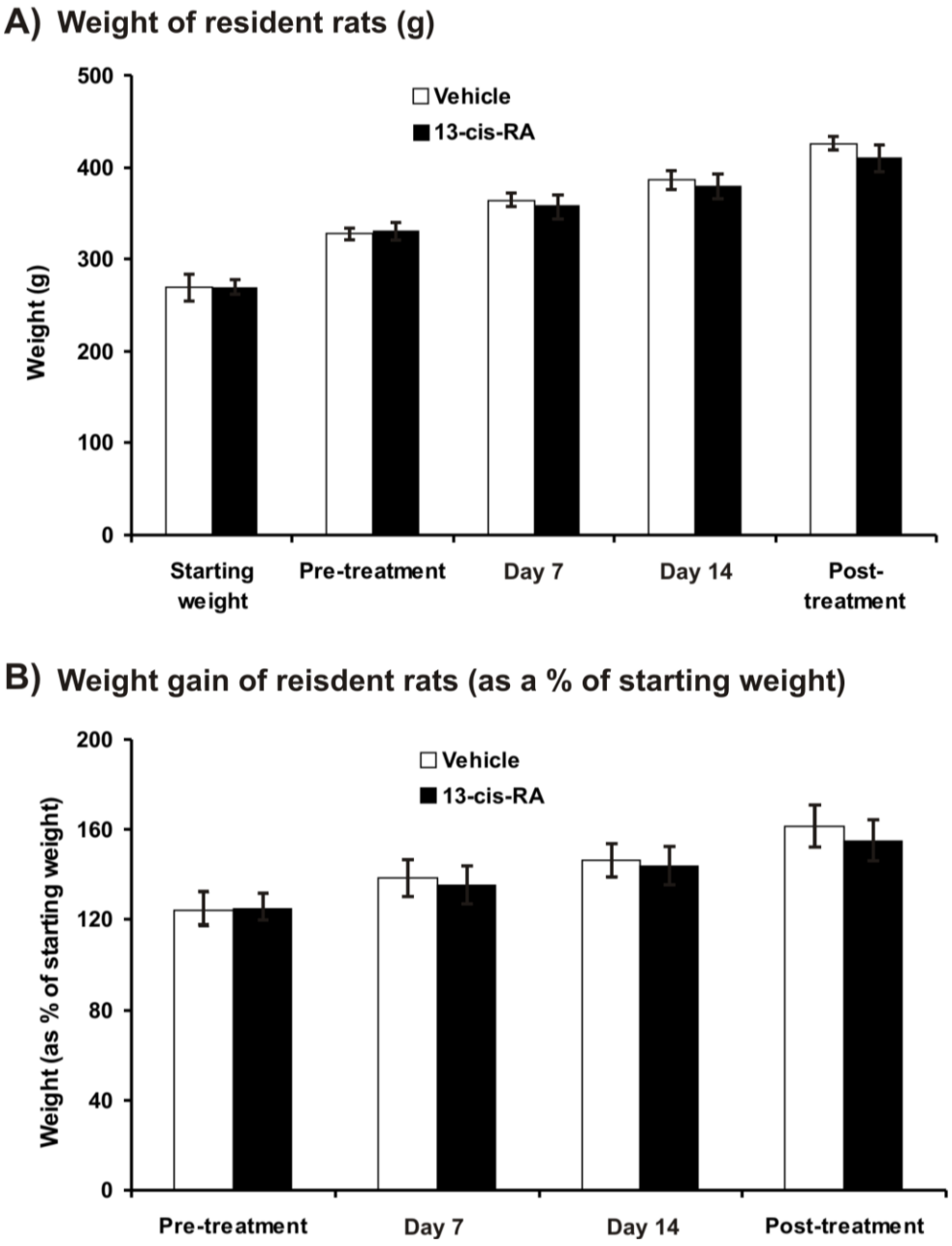

Figure 3.2: Mean group weight and mean group weight gain as a percentage of starting weight of resident-rats during the resident-intruder paradigm. A) Both vehicle (open bars) and 13-*cis*-RA-treated resident rats (closed bars, n=8/group) were weighed once a week, with steady increases in weight measured. B) Weight gain was measured as the measured weight relative to the rats starting weight and steady increases were recorded, indicating good general health and low levels of stress.

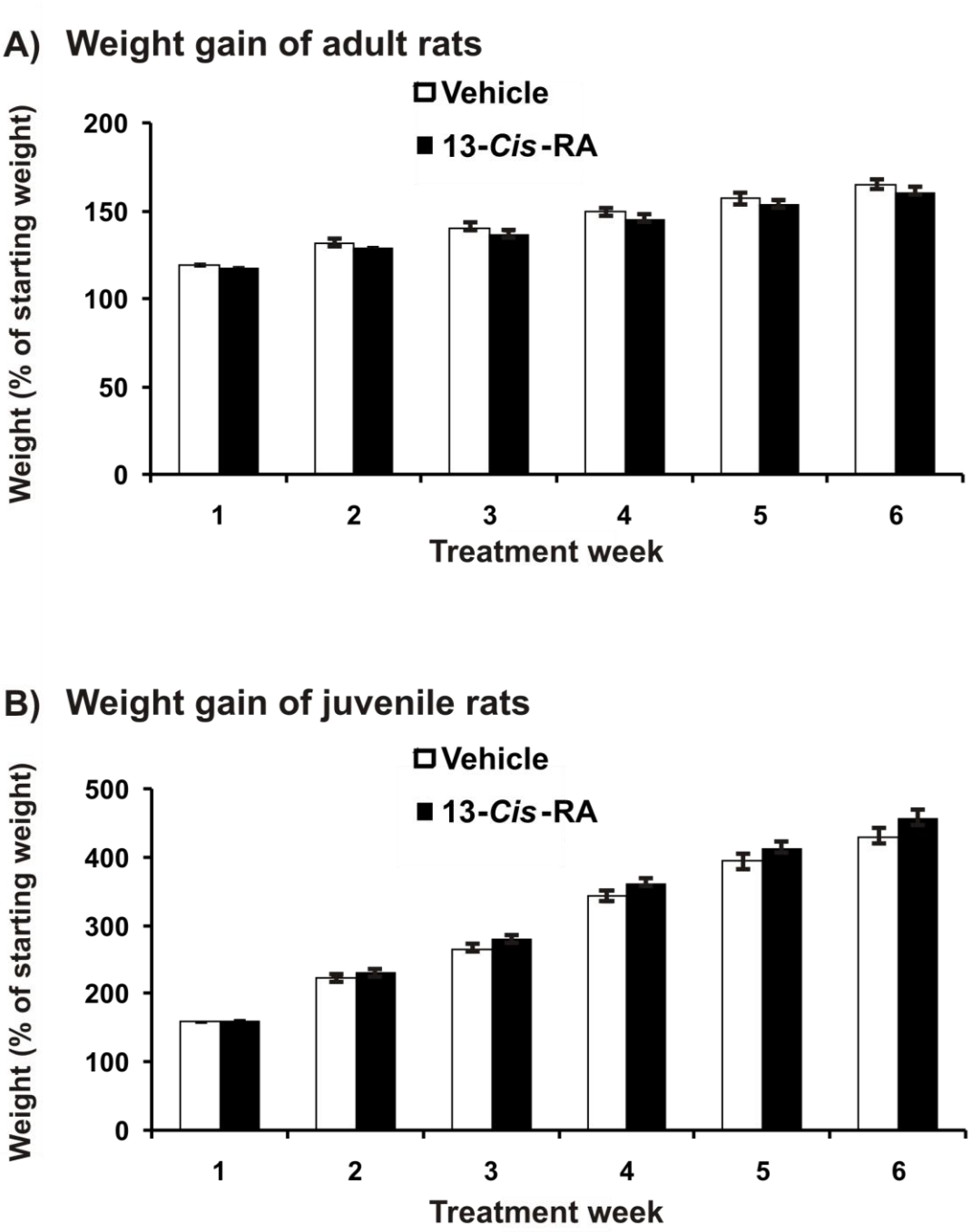

Figure 3.3: Weight gain of adult (A) and juvenile (B) rats undergoing chronic (6 wk) treatment with either vehicle (open bars) or 13-*cis*-RA (closed bars, n=8/group). A) Adult rats treated with either vehicle or 13-*cis*-RA display consistent weight gain and likewise in B) juvenile rats gain weight consistently throughout chronic treatment with both vehicle and 13-*cis*-RA.

3.3.2. Effects of 13-*cis*-RA on resident rat behaviour in the resident-intruder paradigm

The analysis of the social behaviour between unfamiliar intruder rats and resident rats treated with vehicle or 13-*cis*-RA are summarised in Table 3.3 and Figure 3.4.

Behaviour	**Day 0 (pre-treatment)**	**Day 7**	**Day 14**	**Day 21 (post-treatment)**
Vehicle-treated rats				
Exploration	23.4 ± 1.9	19.0 ± 1.6	22.5 ± 1.4	24.1 ± 2.1
Maintenance	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.3	2.1 ± 0.8
Investigation	50.0 ± 0.9	50.6 ± 2. 0	47.7 ± 1.0	47.8 ± 1.8
Sexual	0.7 ± 0.4	1.2 ± 0.5	0.2 ± 0.1	0.3 ± 0.1
Aggression	11.1 ± 1.5	15.4 ± 0.8*	13.0 ± 1.1	19.8 ± 1.2
Flight Submit	0.6 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.4
Flight Escape	13.3 ± 0.5	12.1 ± 0.7	14.4 ± 0.8	13.9 ± 0.9
Total Behaviours	1677.1 ± 53.5	1706.6 ± 80.5	1631.3 ± 41.8	1500.9 ± 74.5
13-*Cis*-RA-treated rats				
Exploration	29.6 ± 2.1	23.1 ± 1.4*	25.5 ± 1.1	22.9 ± 1.5*
Maintenance	0.8 ± 0.3	0.8 ± 0.2	1.5 ± 0.4	0.9 ± 0.4
Investigation	48.2 ± 1.4	48.8 ± 1.7	45.6 ± 1.3	50.3 ± 1.6
Sexual	0.6 ± 0.4	1.7 ± 0.5	0.7 ± 0.3	0.5 ± 0.2
Aggression	7.6 ± 0.8	4.7 ± 0.7* ¥¥	4.5 ± 0.8* ¥¥	10.1 ± 1.0††
Flight Submit	0.7 ± 0.2	3.3 ± 0.6** ¥¥	2.8 ± 0.4** ¥¥	2.0 ± 0.7
Flight Escape	12.6 ± 0.3	17.6 ± 0.9** ¥¥	19.4 ± 0.9** ¥¥	13.2 ± 0.6††
Total Behaviours	1527.1 ± 91.3	1610.3 ± 65.8	1589.8 ± 48.5	1585.5 ± 44.7

Table 3.3: Analysis of 13-*cis*-RA-treated resident rat behaviour on each of four encounters (days 0, 7, 14, and 21) in the resident–intruder paradigm. Values indicate mean ± SEM percentage of total behaviour score, except total behaviour score=mean ± SEM absolute observations. * indicates P<0.05, ** indicates P<0.01 c.f. day 0 (Pre-dose). †† denotes P<0.01 c.f. Day 14, while ¥¥ denotes P<0.01 c.f. vehicle-treated resident rats.

1-Way ANOVA with repeated measures revealed significant main effects of treatment on aggressive [$F(1,14)=54.661$, $P<0.0001$], flight submit [$F(1,14)=12.195$, $P=0.0036$] and flight escape [$F(1,14)=10.900$, $P=0.0052$] behaviours but not on any of the other categories of behaviour nor total behaviour score [all $Fs(1,14)\leq 3.533$, $P\geq 0.0811$ in all cases]. Furthermore, analysis revealed

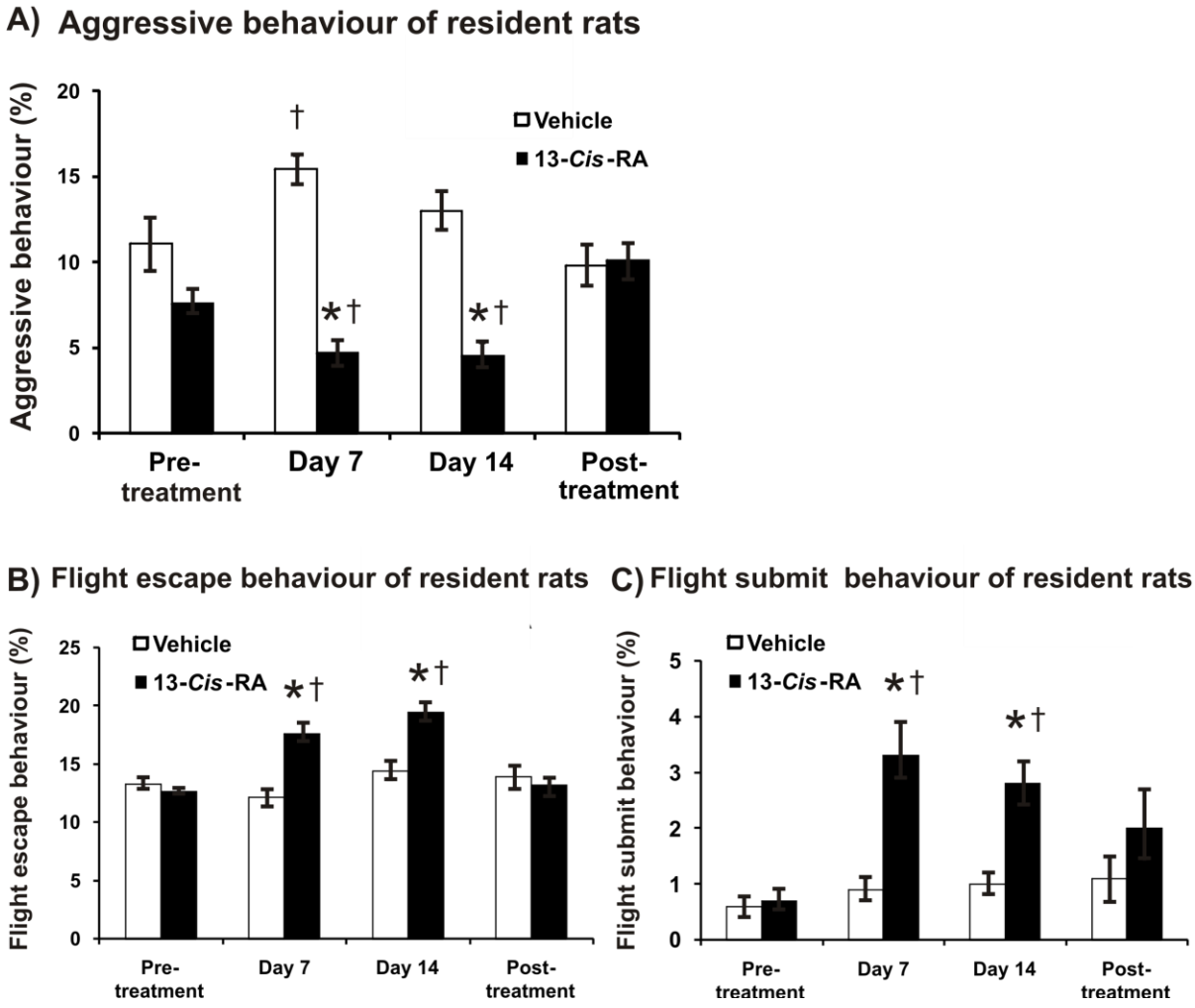

Figure 3.4: Ethological analysis of resident rat behaviour in the resident-intruder paradigm following 13-*cis*-RA treatment. Vehicle (n=8, open bars) or 13-*cis*-RA-treated (n=8, closed bars) adult resident rats encountered intruder rats on each of four weekly occasions (day 0=pretreatment, day 7=7 days of treatment, day 14=14 days of treatment, day 21=post-treatment). (A) Drug-treated resident rats displayed altered aggression, with a reduction of the number of aggression behaviours (as a percentage of all behaviours) towards the intruder rat compared with vehicle-treated control resident rats. Concomitant increases in flight escape behaviours (B) and in flight behaviours (C) were also found. Data are mean ± SEM of n=8 resident rats per treatment group. In all graphs significant effects of 13-*cis*-RA treatment compared with vehicle are indicated (*= $P<0.05$). Within treatment groups, significant differences in behaviours compared with pre-treatment baseline values (Day 0) are also indicated (+ =$P<0.05$).

significant main effects of time on exploration [$F(3,42) = 5.047$, $P=0.0045$], flight submit [$F(3,42) = 7.431$, $P=0.0004$], flight escape [$F(3,42)=13.660$, $P<0.0001$] and sexual [$F(3,42)=6.267$, $P=0.0013$] behaviours but not on any of the other categories of behaviour nor total behaviour score [all Fs $(3,42)\leq 2.135$, $P\geq 0.1101$ in all cases]. Finally, significant treatment*time interactions were identified for aggression [$F(3,42)=13.178$, $P<0.0001$], flight submit[$F(3,42)=3.279$, $P=0.0301$] and flight escape [$F(3,42)=14.003$, $P<0.0001$] behaviours but not on any of the other categories of behaviour nor total behaviour score [all Fs$(1,14)\leq 2.310$, $P\geq 0.0901$ in all cases].

Within-treatment comparisons revealed that resident rats treated with vehicle exhibited increased aggressive behaviour on day 7 of treatment compared to the level of aggression observed prior to treatment (i.e. day 0; $P<0.05$). No other significant differences in aggressive, exploration, flight submit, flight escape or sexual behaviours were observed in these control rats throughout the experiment (adjusted Ps>0.05 in all cases). Within-treatment comparisons also revealed that at days 7 and 14 of treatment, resident rats treated with 13-*cis*-RA exhibited significantly reduced aggressive behaviour (adjusted Ps<0.05 in both cases), concomitant with increased flight submit and flight escape behaviour (adjusted Ps<0.01 in all cases, Figures 3.4A, B & C).

By 7 days following the cessation of 13-*cis*-RA treatment the levels of aggression, flight submit and flight escape behaviours had generally returned to baseline (day 0 compared to post-dose day 7; adjusted $P>0.05$ in all cases, Figure 3.4). Consequently, at post-treatment day 7 resident rats in the 13-*cis*-RA group exhibited increased aggression with reduced flight escape behaviour compared to the respective levels observed at day 14 of treatment ($P<0.01$ in both cases). Further post-hoc analysis revealed that 13-*cis*-RA-treated resident rats exhibited reduced exploration at day 7 (but not day 14) of treatment and 7 days following treatment ($P<0.05$ in both cases). No significant changes in sexual behaviour (including attempted mount and penis licking) were observed throughout the course of the experiment in 13-*cis*-RA-treated resident rats, although it should be noted that the full range of sexual behaviours (including full mount) were not analysed as all the resident and intruder rats were male.

3.3.3. Effects of 13-*cis*-RA on behaviour in the forced swim test

The FST was preceded by a 15 min pre-swim session 24 hours earlier (as per (Detke *et al.*, 1995; Lucki, 1997)) and the total amount of time spent in swimming, climbing or immobility behaviours during the first 6 min of this pre-swim session are shown in Figure 3.5. There was no significant difference in the behaviour of adult (Figure 3.5A,B) or juvenile (Figure 3.5C,D) rats treated with 13-*cis*-RA compared to vehicle -treated control animals at both week 2 (Figure 3.5A,C) and week 6 (Figure 3.5B,D) of 13-*cis*-RA treatment (two-way ANOVA, $P<0.05$).

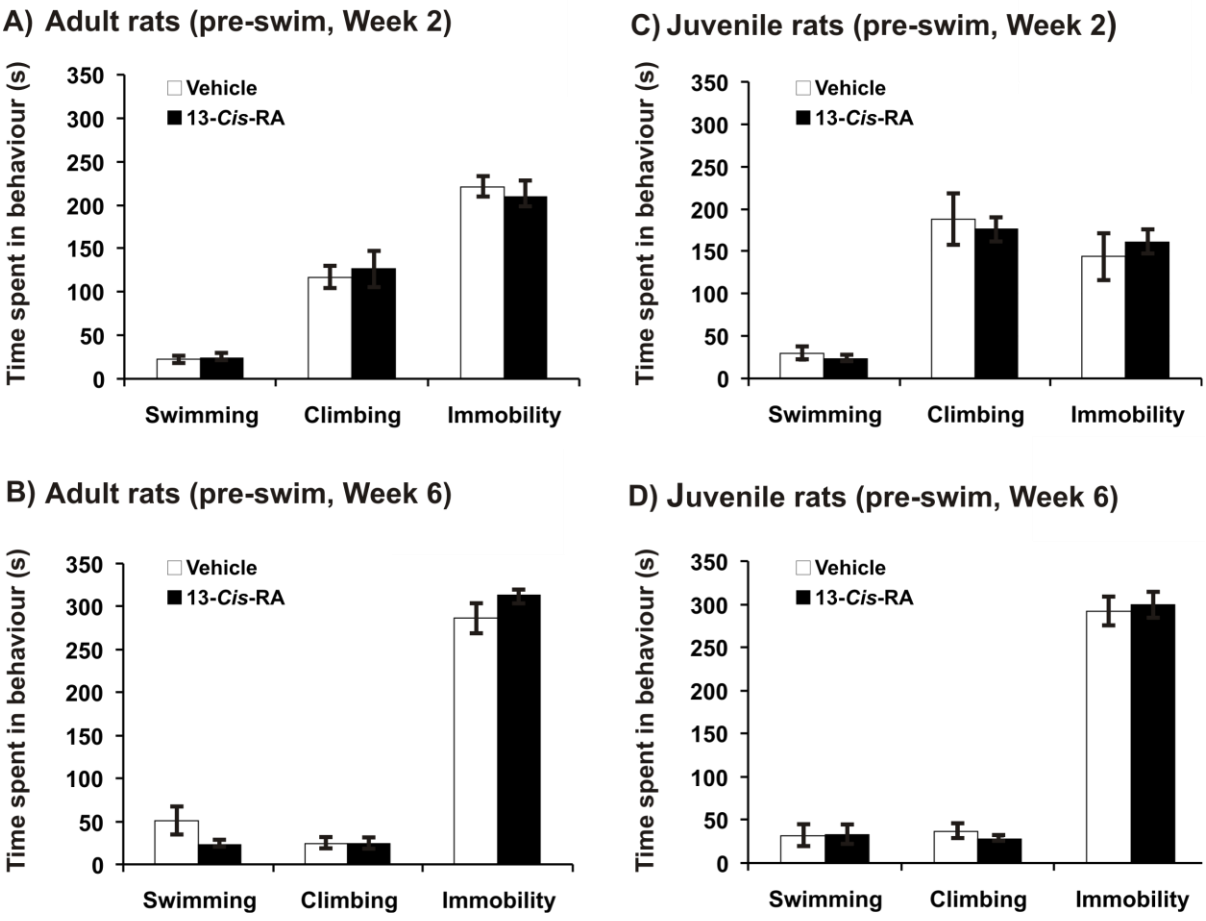

Figure 3.5: Analysis of pre-swim test session (as part of the FST) following 13-*cis*-RA treatment. (A) The performance of adult rats in the pre-swim session was analysed after two weeks of either vehicle (n=8, open bars) or 13-*cis*-RA (n=8, closed bars) treatment, while performance after 6 weeks of treatment is shown in (B). Graph (C) shows pre-swim performance in juvenile rats after two weeks treatment with either vehicle (n=8, open bars) or 13-*cis*-RA (n=8, closed bars), while group (D) shows juvenile rat performance after 6 weeks of treatment.

Following the pre-swim session, the same group of rats underwent the 6 min FST session 24 h later to determine depression-related behaviour (shown in Figure 3.6). The total amount of time spent in swimming, climbing or immobility behaviours during the 6 min test session were measured and no significant difference was found in the behaviour of adult (Figure 3.6A,B) or juvenile (Figure 3.6C,D) rats treated with 13-*cis*-RA compared to vehicle-treated control animals. Two-way ANOVA was used to analyse the total time spent immobile and there was no significant main effect of age [F(1,7)=0.381, *P*=0.557] or any interaction between age and treatment [F(3,21) = 1.281, *P*=0.328]. A significant main effect of treatment with repeated measures was revealed [F(3,21)=14.672, *P*<0.001], although post-hoc analysis revealed that there was no significant effect of 13-*cis*-RA treatment.

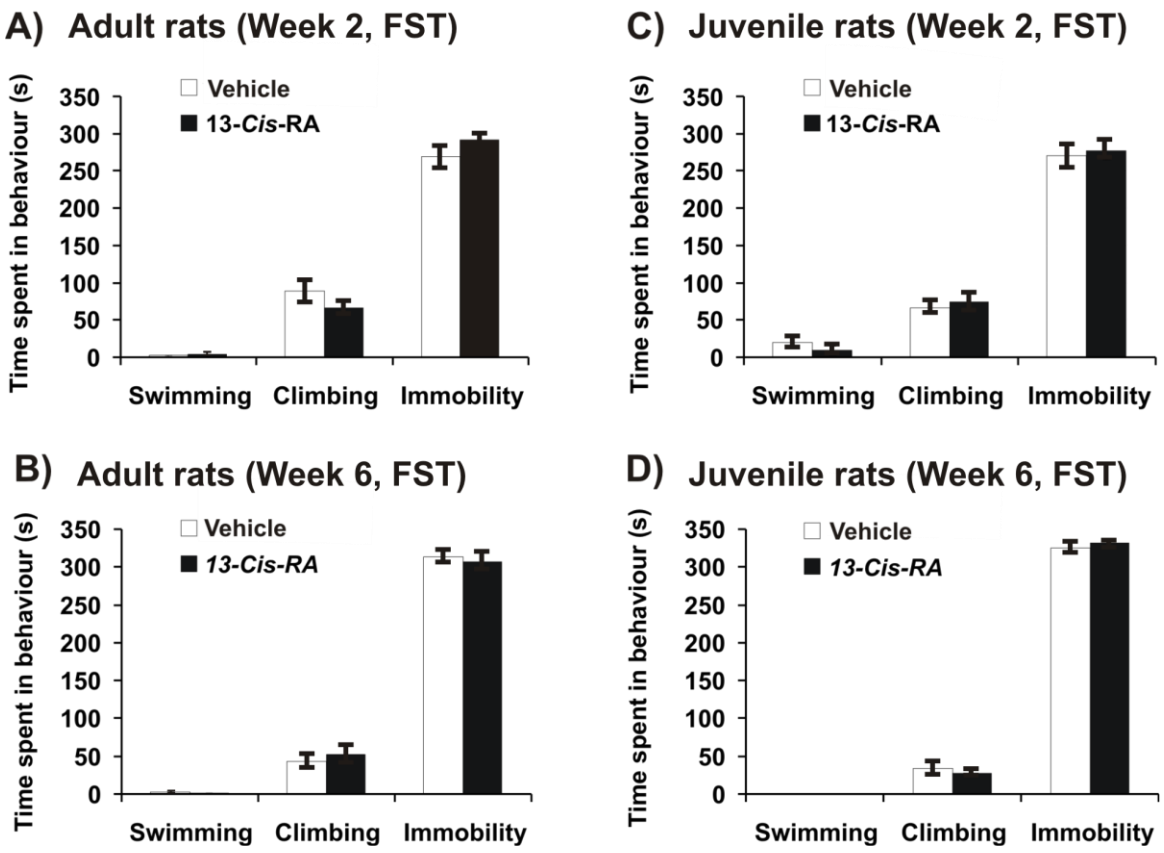

Figure 3.6: Behaviours exhibited during the FST session following 13-*cis*-RA treatment. (A) Analysis of adult rat swimming, climbing and immobility behaviour after two weeks of treatment with either vehicle (n=8, open bars) or 13-*cis*-RA (n=8, closed bars). (B) The same group of rats tested after 6 weeks of treatment. (C) FST performance after two weeks of treatment with either vehicle (n=8) or 13-*cis*-RA (n=8) in juvenile rats, while (D) shows the same juvenile groups tested after 6 weeks of treatment.

However, repeated testing at 6 weeks compared to 2 weeks significantly increased the time spent immobile in both adult and juvenile rats regardless of treatment (P<0.05 in all cases, 1-way ANOVA). In adult rats tested after treatment for 2 weeks (Figure 3.6A), the mean time spent immobile for vehicle-treated animals was 269 ± 14 s and with 13-*cis*-RA 292 ± 8 s, n=8 per group. After 6 weeks treatment (Figure3.6B), the mean time spent immobile for vehicle-treated animals increased to 315 ± 7 s and with 13-*cis*-RA to 306 ± 11 s, n=8 per group. In juvenile rats, there was also a significant effect of repeated testing (P<0.05 in all cases, 1-way ANOVA) when the data after 2 weeks of treatment (mean time spent immobile for vehicle: 272 ± 12 s and 13-*cis*-RA 272 ± 13 s, n=8) was compared with data after 6 weeks treatment (mean time spent immobile for vehicle: 326 ± 7 s and 13-*cis*-RA 332 ± 4 s, n=8).

Immobility times were further analysed by comparing pre-swim performance with FST performance at 2 and 6 weeks of treatment in both adults (Figure 3.7A) and juveniles (Figure3.7B). The time spent immobile during the pre-swim session showed no significant main effect of treatment or any interaction between age and treatment [Fs(3,21)≤1.152, P≥0.287] (Figure 3.7A,B). However, there was a significant main effect of age [F(1,7)=10.239, P=0.018] such that juvenile animals exhibited behaviours that were different to those of the adult animals during the pre-swim test session at 2 weeks for both vehicle and 13-*cis*-RA-treated groups (shown in Figure 3.8). Post-hoc analysis revealed that during the first 6 min of the initial pre-swim session juvenile animals spent significantly less time immobile than adult animals (mean time spent immobile juvenile vs adult vehicle-treated: 143.5 ± 27.0 s vs 221.1 ± 11.5 s and juvenile vs adult 13-*cis*-RA-treated: 161.1 ± 13.7 s vs 209.5 ± 17.5 s n=8 per group, P<0.04).

3.3.4. Effects of 13-*cis*-RA on sucrose consumption

Preliminary sucrose consumption experiments were performed whereby the optimal concentration of sucrose solution and optimal observation time of sucrose solution consumption were determined. The concentration of sucrose solution was determined given that different strains of rats appear to prefer

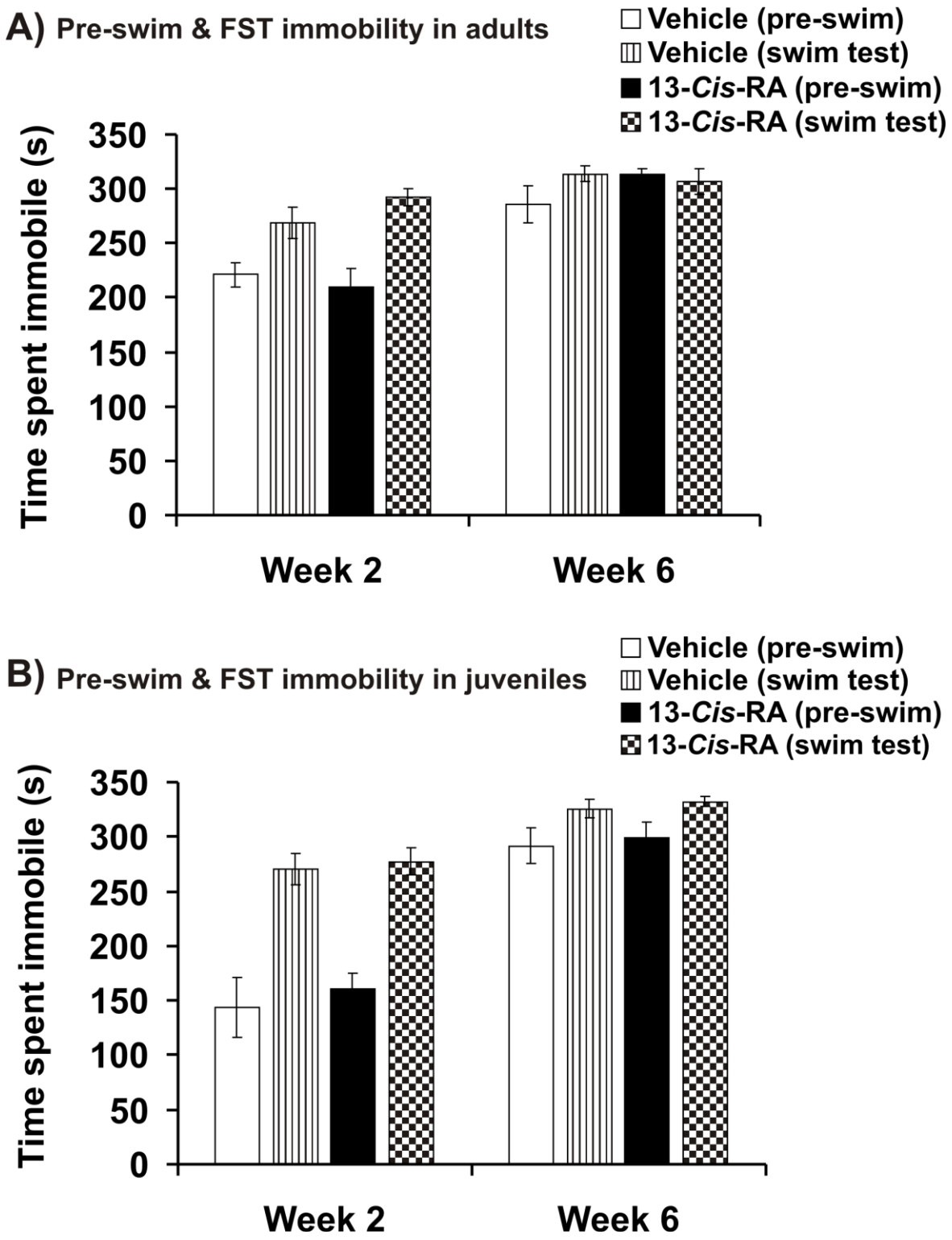

Figure 3.7: Comparison of immobility times from pre-swim and subsequent FST session in 13-*cis*-RA-treated adult rats (A) and juveniles (B). The pre-swim immobility times of adults (n=8/group) at week 2 are significantly higher than the pre-swim immobility times of juveniles (n=8/group) at week 2; an effect that is not seen by week 6 pre-swim performances. The performances of the FST session at 2 and 6 wks do not differ between adults and juveniles.

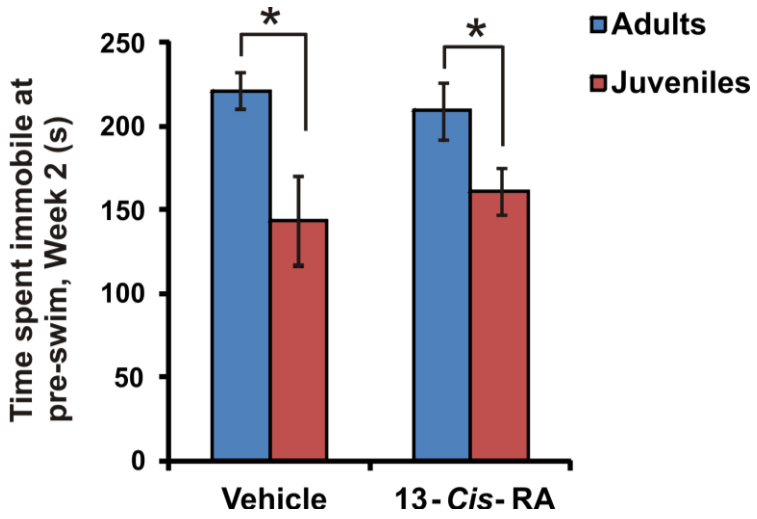

Figure 3.8: Comparison of immobility times from pre-swim (Week 2) in vehicle and 13-*cis*-RA-treated adult and juvenile rats. The pre-swim immobility times of adults (blue bars, n=8/group) at week 2 are significantly higher than corresponding juveniles (red bars, n=8/group), irrespective of vehicle or 13-*cis*-RA treatment. No differences were seen in Week 2 FST behaviour, or Week 6 pre-swim and FST behaviour. * denotes $P<0.04$

different sucrose concentrations (Lush, 1989; Willner, 1997), with inbred PVG rats and Lister hooded rats preferring a 1% concentration and Wistar rats generally preferring a 2% concentration.

Furthermore, the concentration of sucrose solution is thought to affect the ability of chronic mild stress to reduce sucrose solution consumption (Muscat *et al.*, 1991; Willner, 1991). High sucrose consumption rates (compared with water) indicate that the solution is pleasurable and increases the reliability of the experiment. In addition, the length of time over which consumption rates were recorded was altered to test the optimum duration required to observe greater sucrose solution consumption compared with water. After 16 h of food and water deprivation (overnight), adult rats were given water or two concentrations of sucrose solution (1% w/v or 3% w/v, dissolved in water, n=3/group) and consumption rates were measured as g/kg over 1 and 2 hours (shown in Figure 3.9).

Following overnight food and water deprivation, rats drank 11.08 ± 9.52g/kg and 25.22 ± 11.24g/kg of water over the course of 1 and 2 hours, respectively. A 1% sucrose solution showed a trend to double the amount of solution consumed over 2 hours to 51.27 ± 6.86g/kg (P=0.11), while 3% sucrose solution did not significantly increase the amount of sucrose consumed (P=0.21).

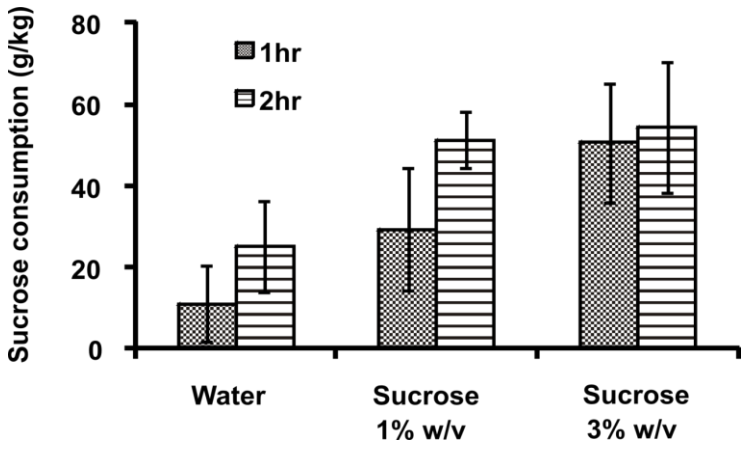

Figure 3.9: Preliminary sucrose consumption test data. Adult rats were food and water deprived overnight (16 h) and then given either water (n=3), sucrose 1% w/v (n=3) or sucrose 3% w/v (n=3). The consumption was measured as grams of solution consumed per kilogram of body weight of each rat for both 1 and 2 h.

In subsequent sucrose consumption experiments 1% sucrose solution was used and a 2 h test period employed to behaviourally test 13-*cis*-RA-treated rats.

The consumption of sucrose solution (following 16 h food and water deprivation) of adult and juvenile rats treated with either vehicle or 13-*cis*-RA daily for 2 and 6 weeks is shown in Figure 3.10. A two-way ANOVA was used to analyse the total sucrose consumption after 1 h (Figure 3.10A) and after 2 h (Figure 3.10B) and for both measures, there was a significant main effect of age [$Fs(1,7)>14.590$, $P<0.007$] although there was no significant main effect of treatment or any interaction between age and treatment [$Fs(3,21)<3.357$, $P>0.130$]. Post-hoc analysis using one-way ANOVA revealed that when animals were tested after 2 weeks of treatment juvenile animals consumed more sucrose solution than adult animals (mean sucrose consumption (2 h) for vehicle-treated adult: 57.4 ± 2.8g/kg and juvenile: 91.5 ± 7.6g/kg, $P<0.001$, n=8). The effect was regardless of treatment as mean sucrose consumption (2 h) for 13-*cis*-RA-treated adult and juvenile rats was 64.1 ± 5.2g/kg and 92.2 ± 8.8g/kg, respectively ($P<0.01$, n=8). While this trend was maintained after 6 weeks of treatment there was no significant difference between juvenile and adult animals at this time point.

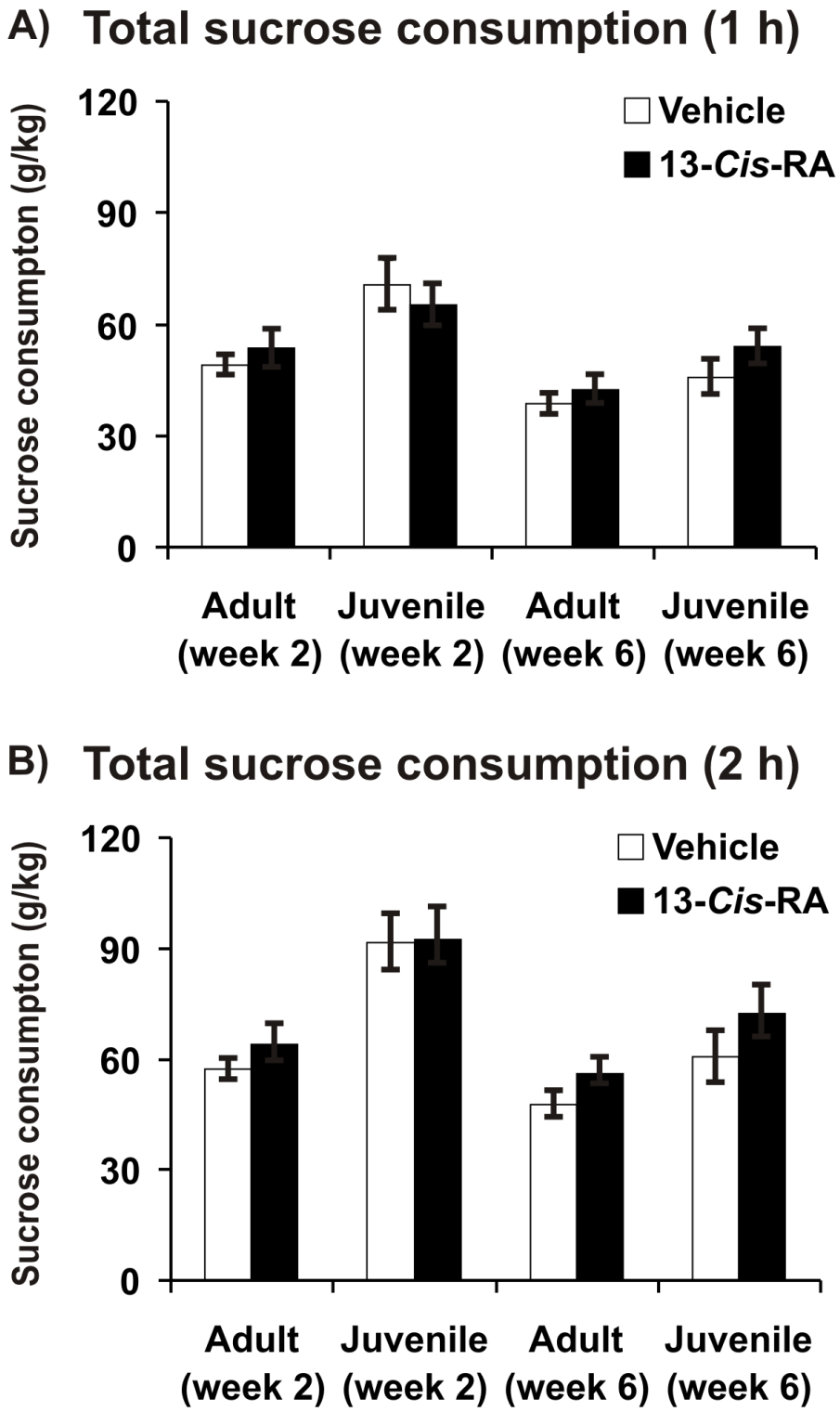

Figure 3.10: The effect of 13-*cis*-RA treatment on sucrose consumption in the adult and juvenile rats. The mean total sucrose consumption (corrected for body weight) during a 1 h (A) and 2 h (B) test session is shown. (A) Both adult and juvenile rats were treated with either vehicle or 13-*cis*-RA (n=8/group). Levels of sucrose consumption were measured after 2 weeks or 6 weeks of treatment and there was no significant effect of 13-*cis*-RA (closed bars) compared with vehicle controls (open bars). Data shown are mean ± SEM of n=8.

3.3.5. Effects of 13-*cis*-RA on locomotor behaviour in the open field

To determine whether any behavioural effects of 13-*cis*-RA treatment could be attributed to a change in locomotor activity, rats were also tested in the open field. Two-way ANOVA was used to analyse the total number of line crossings (Figure 3.11A) and the number of vertical rears (Figure 3.11B) for each group of animals. For both measures, there was a significant main effect of age [$Fs(1,7) \geq 14.920$, $P \leq 0.006$] and a significant main effect of treatment with repeated measures [$Fs(3,21) \geq 11.460$, $P \leq 0.015$] but no significant interaction between age and treatment [$Fs(3,21) \leq 4.287$, $P \geq 0.120$].

Post-hoc analysis using one-way ANOVA revealed that, regardless of treatment, the mean total number of line crossings was significantly higher in juvenile treatment groups than in adult animals after both 2 weeks (the mean number of line crossings for vehicle-treated adult and juvenile rats was 108.9 ± 7.3 and 136.9 ± 7.4, respectively and for 13-*cis*-RA-treated adult and juvenile rats was 106.9 ± 8.7 and 135.0 ± 8.5, respectively, $P<0.002$, n=8 per group) and 6 weeks of treatment (the mean number of line crossings for vehicle-treated adult and juvenile rats was 59.6 ± 4.9 and 88.1 ± 5.4, respectively and for 13-*cis*-RA-treated adult and juvenile rats was 71.5 ± 6.0 and 94.1 ± 7.3, respectively, $P<0.03$, n=8 per group). Furthermore, there was a significant effect of repeated testing such that in all groups, regardless of treatment or age, locomotor activity was reduced when tested after 6 weeks treatment to 60–70% of the activity levels recorded after 2 weeks treatment ($P<0.005$, paired t-test).

Similarly, post-hoc analysis of vertical rearing behaviour in the open field (Figure 3.11B) revealed that, in general, juvenile animals made significantly more vertical rears than adult animals after both 2 weeks (the mean number of vertical rears for 13-*cis*-RA-treated adult and juvenile rats was 47.3 ± 5.0 and 64.0 ± 3.7, respectively, $P=0.04$, n=8 per group) and 6 weeks of treatment (the mean number of vertical rears for vehicle-treated adult and juvenile treated rats was 34.5 ± 5.5 and 49.4 ± 3.9, respectively, $P=0.02$ and for 13-*cis*-RA-treated adult and juvenile rats was 29.5 ± 3.2 and 53.3 ± 3.8, respectively, $P<0.001$, n=8 per group).

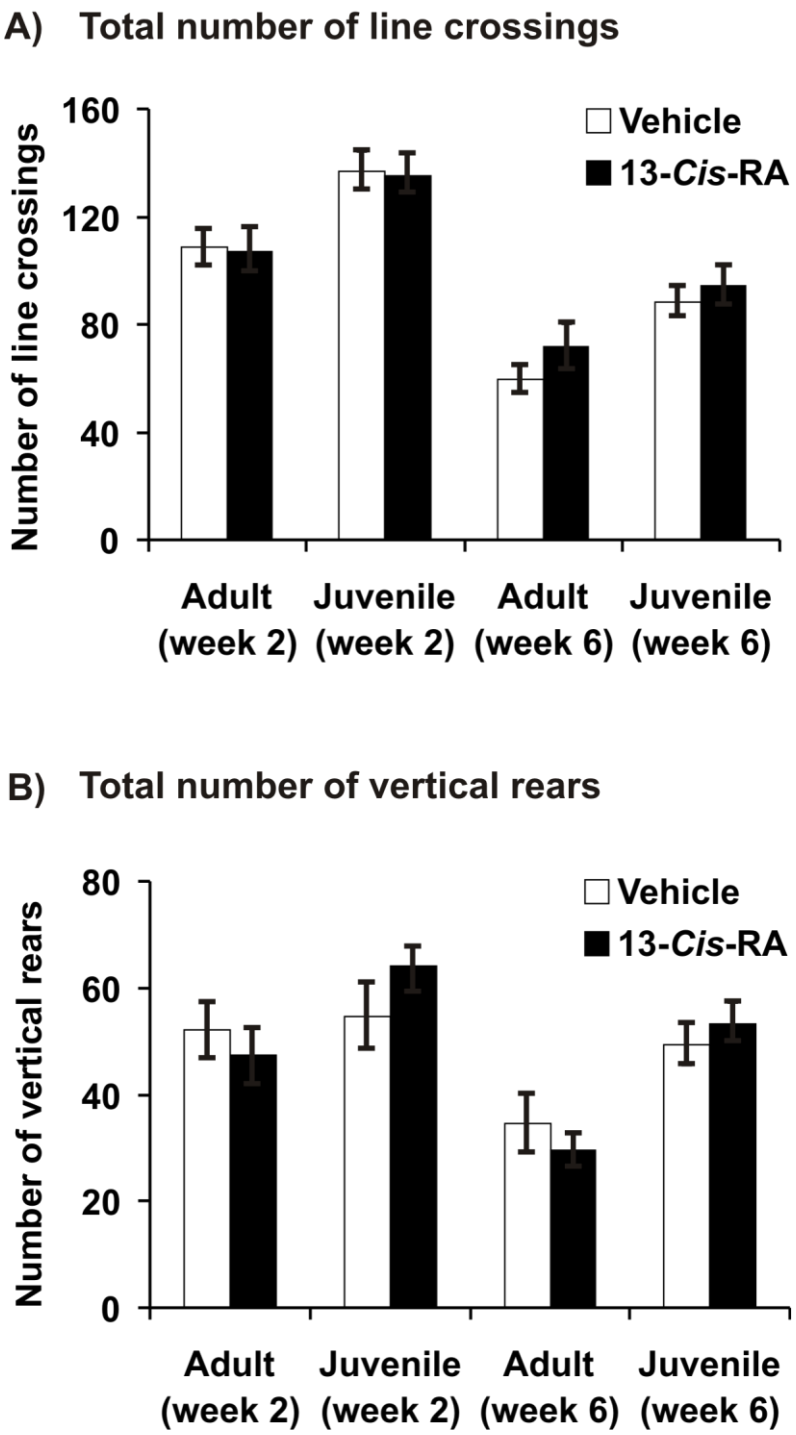

Figure 3.11: Locomotor activity of adult and juvenile rats measured in the open field test. Locomotor activity was assessed by the total number of line crossings (A) and the number of vertical rears (B) in the open field during a 10 min test session. Both adult and juvenile rats were treated with either 13-*cis*-RA (closed bars) or vehicle (open bars). There was no significant effect of drug on locomotor activity compared with vehicle rats (n=8/group). Data shown are mean ± SEM of n=8 rats per treatment group.

In addition, there was a significant effect of repeated testing but only in adult animals, such that exposure to the open field arena after 6 weeks of treatment significantly reduced vertical rearing behaviour compared with 2 weeks in both vehicle and 13-*cis*-RA treatment groups ($P<0.005$, two-way ANOVA). The reduction in locomotion is likely to reflect the decreased novelty of the open field test experienced by the animal upon repeated exposure (as seen by (Karrenbauer *et al.*, 2009) and others).

3.3.6. The effects of 13-*cis*-RA on 8-OH-DPAT-induced hypothermia

Preliminary hypothermia experiments were undertaken to determine the dose of 8-OH-DPAT and WAY-100635 required to achieve measurable levels of hypothermia in Wistar rats and its blockade, respectively. Starting doses of 8-OH-DPAT were based on previous studies (Bill *et al.*, 1991; Knapp *et al.*, 1998) showing that ~0.5mg/kg (s.c.) induced maximal decreases in rat body temperature after 30 min, whereas 0.3mg/kg (s.c.) induced a measurable sub-maximal decrease after 30 min. These results were replicated in Figure 3.12A, whereby 0.5mg/kg and a sub-maximal dose of 0.3mg/kg of 8-OH-DPAT lead to a 2.43 ± 0.13°C and 2.33 ± 0.08°C reduction in basal body temperature (n=2/group, adult rats). Administration of 0.1mg/kg WAY-100635, a 5-HT_{1A}R antagonist, 30 min prior to 0.5mg/kg 8-OH-DPAT administration (Forster *et al.*, 1995) was sufficient to attenuate 8-OH-DPAT-induced hypothermia (Figure 3.12B, n=2, adult rats). Based on these findings, subsequent hypothermia experiments used a sub-maximal dose of 0.3mg/kg 8-OH-DPAT as it allowed for both a potential increase/decrease in magnitude of hypothermia while remaining measurable. A dose of 0.1mg/kg for WAY-100635 was also subsequently used to confirm the involvement of 5-HT_{1A}R s in 8-OH-DPAT induced hypothermia.

8-OH-DPAT- induced hypothermic responses were analysed in adult rats following 6 weeks of 13-*cis*-RA treatment. Figure 3.13A shows the absolute body temperatures recorded in both vehicle and 13-*cis*-RA-treated rats that had received either 8-OH-DPAT or saline (n=6/group) and Figure 3.13B shows relative body temperatures from the individual baseline temperatures. Firstly, all treatment groups of rats had similar baseline temperatures. The vehicle + 8-OH-DPAT and 13-*cis*-RA + 8-OH-

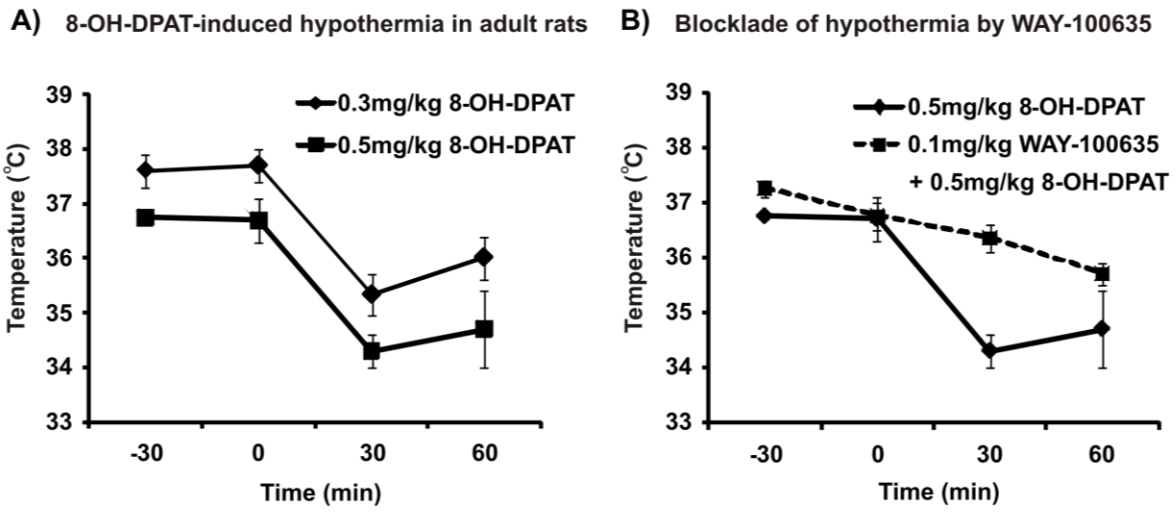

Figure 3.12: Preliminary 8-OH-DPAT-induced hypothermia experiments. (A) Adult rats were injected s.c. with 0.3 or 0.5mg/kg of 8-OH-DPAT in a volume of 2.5ml/kg saline at t=0 min and subsequent hypothermia was recorded. (B) Adult rats received 0.1mg/kg of WAY-100635 (t=-30min) 30 min prior to 0.5mg/kg of 8-OH-DPAT (t=0 min) and subsequent blockade of hypothermia was recorded. Values are mean ± SEM and n=2/gp.

DPAT-treated rats had baseline temperatures (averaged from readings at t= -30 min and t= 0 min) of 34.23 ± 0.08 ºC and 34.64 ± 0.09 ºC respectively, whereas vehicle + saline and 13-*cis*-RA + saline-treated rats had baseline temperatures of 34.88 ± 0.09 ºC and 34.91 ± 0.07 ºC, respectively (Figure 3.13A). Hypothermic changes in body temperature were then observed in rats that had received 8-OH-DPAT but not saline, irrespective of vehicle or 13-*cis*-RA treatment (vehicle + 8-OH-DPAT-treated rats had a significantly lower body temperature than the vehicle + saline-treated rats, $F[1,10]=4.94$, $P=0.05$, and likewise 13-*cis*-RA + 8-OH-DPAT-treated rats had significantly lower body temperatures than the 13-*cis*-RA + saline groups, $F[1,10]=9.64$, $P=0.01$, across all time-points). 13-*Cis*-RA treatment had no effect of the level of hypothermia induced in 8-OH-DPAT-treated rats (vehicle + 8-OH-DPAT-treated rats compared with 13-*cis*-RA+ 8-OH-DPAT-treated rats, $F[1,10]=0.07$, $P=0.791$) or any change in body temperature in saline-treated rats (vehicle + saline-treated rats compared with 13-*cis*-RA+ saline-treated rats, $F[1,10]=0.111$, $P=0.745$, across all time-points). The greatest divergence between vehicle and 13-*cis*-RA-treated rats occurred in the 8-OH-DPAT-treated rats at t= 15 min, although this effect was not significant ($P=0.20$).

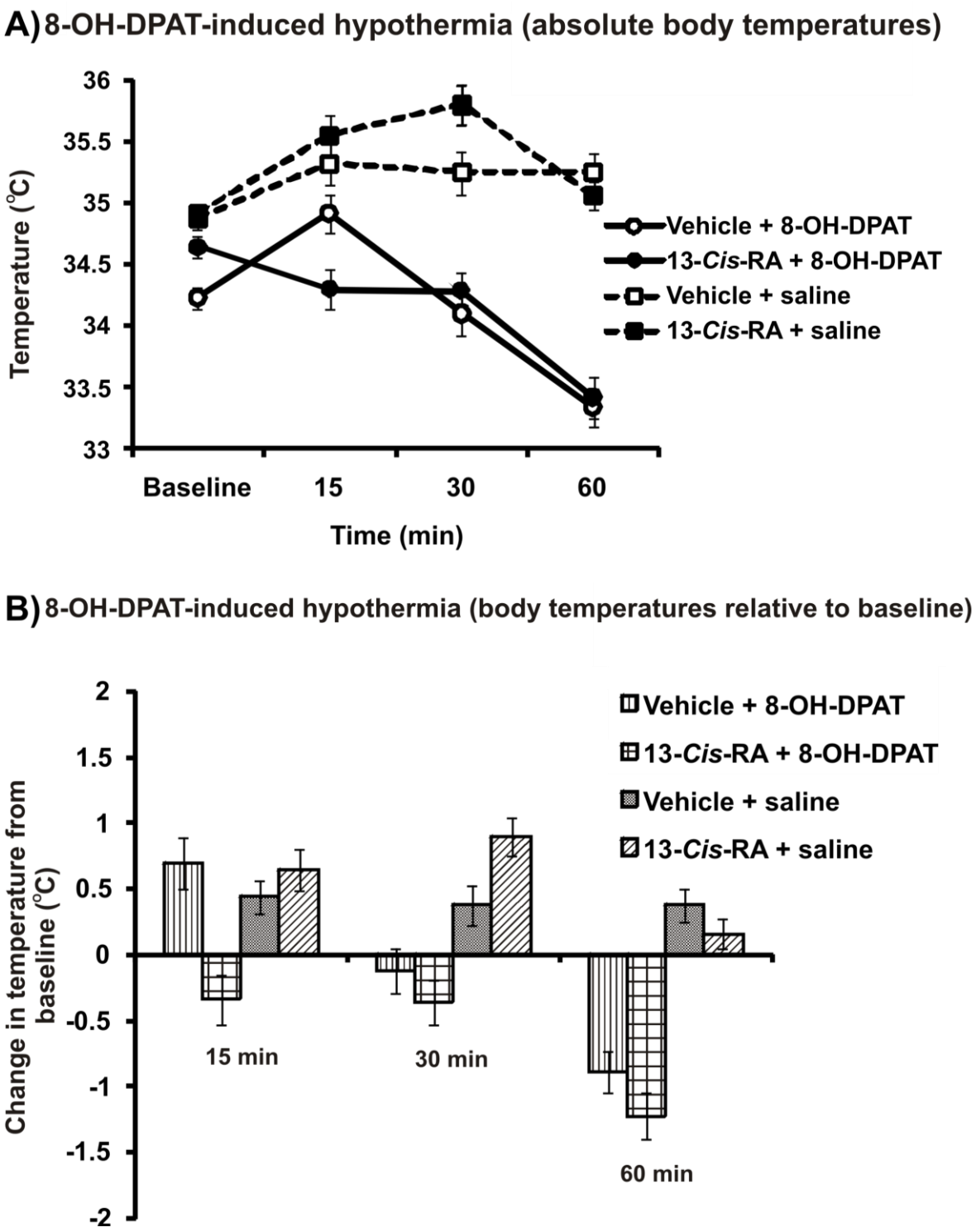

Figure 3.13: 8-OH-DPAT-induced hypothermia in 13-*cis*-RA-treated rats. A) Absolute body temperatures of adult rats that received 6 weeks of either vehicle or 13-*cis*-RA treatment (1mg/kg/day, i.p.) and were subsequently given either saline (2.5ml/kg, s.c.) or 8-OH-DPAT (0.3mg/kg in 2.5ml/kg saline, s.c., n=6/group). (B) The changes in body temperatures were normalised to baseline temperatures.

On the following day, the same cohorts of rats underwent WAY-100635 administration (t= -30 min, n=6/group) followed by 8-OH-DPAT administration (t=0 min, n=6/group), to demonstrate the specificity of 8-OH-DPAT to induce hypothermia through 5-HT_{1A}Rs. Figure 3.14A shows absolute body temperatures whereas Figure 3.14B shows the relative body temperatures from the individual baseline temperatures. All treatment groups had similar baseline body temperatures; body temperatures for vehicle + WAY-100635 + 8-OH-DPAT, 13-*cis*-RA + WAY-100635 + 8-OH-DPAT, vehicle + WAY-100635 + saline and 13-*cis*-RA + WAY-100635 + saline-treated rats were 34.07 ± 0.16°C, 34.25 ± 0.12°C, 33.07 ± 0.12°C and 34.25 ± 0.14°C respectively (Figure 3.14A). The subsequent administration of 8-OH-DPAT (t=0 min) did not induce hypothermia, highlighting the antagonistic effect of WAY-100635 (vehicle + 8-OH-DPAT body temperatures were significantly lower than vehicle + WAY-100635 + 8-OH-DPAT-treated body temperatures, $F[1,10]=10.58$, $P=0.009$, whereas 13-*cis*-RA + 8-OH-DPAT body temperatures were significantly lower than 13-*cis*-RA + WAY-100635 + 8-OH-DPAT-treated body temperatures, $F[1,10]=10.18$, $P=0.01$). In fact, the administration of WAY-100635 appeared to elevate body temperatures from baseline body temperatures (irrespective of the administration of 8-OH-DPAT) and suggests WAY-100635 may block the tonic activation of 5-HT_{1A}Rs and could result in the apparent observation of hyperthermia (Figure 3.14A, B).

The change in body temperature of WAY-100635 + 8-OH-DPAT-treated rats appeared to be irrespective of vehicle or 13-*cis*-RA treatment ($F[1,10]=0.002$, $P=0.969$), although 13-*cis*-RA-treated rats in the WAY-100635 + saline group had significantly lower body temperatures than vehicle-treated rats in the WAY-100635 + saline treatment group ($F[1,10]=6.09$, $P=0.033$). Furthermore, the body temperatures of vehicle-treated rats in the WAY-100635 + 8-OH-DPAT group were significantly lower than the WAY-100635 + saline group ($F[1,10]=5.23$, $P=0.045$), although this effect did not occur in the 13-*cis*-RA-treated rats (13-*cis*-RA + WAY-10035 + 8-OH-DPAT-treated rats did not have a significantly lower body temperature than 13-*cis*-RA + WAY-100635 + saline-treated rats, $F[1,10]=0.23$, $P=0.644$).

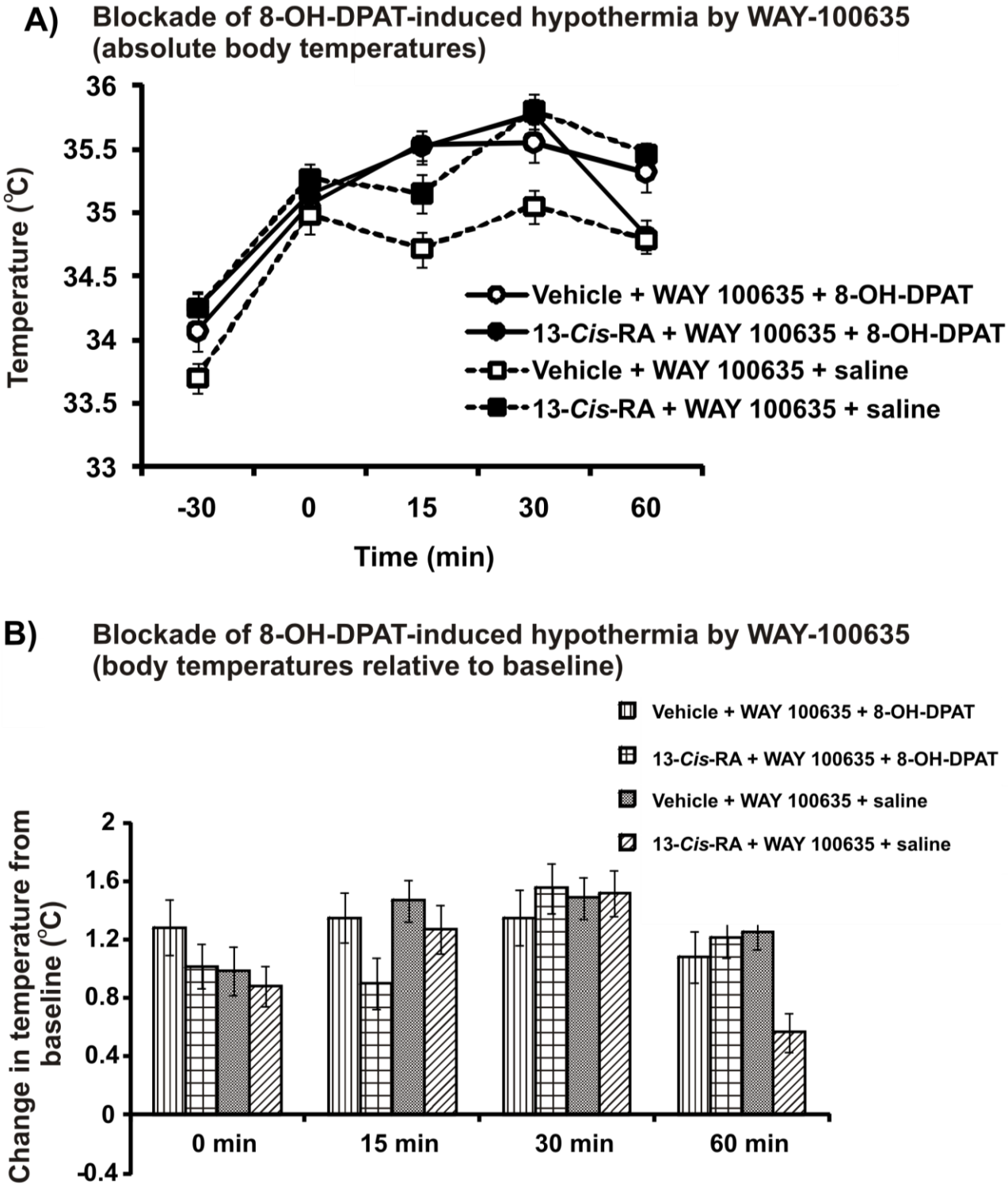

Figure 3.14: The effect of 5-HT_{1A}R antagonism on 8-OH-DPAT-induced hypothermia in 13-*cis*-RA-treated rats. A) Absolute group body temperatures (n=6/group) of rats that had undergone 6 weeks of either vehicle or 13-*cis*-RA treatment (1mg/k/day, i.p.), followed by pretreatment with WAY-100635 (0.1mg/kg in 1ml/kg saline, s.c.) and subsequent administration of either saline (2.5ml/kg, s.c.) or 8-OH-DPAT (0.3mg/kg in 2.5ml/kg saline, s.c.). B) The changes in body temperatures were normalised to baseline temperatures.

3.3.7. 13-*cis*-RA plasma levels

All rats underwent either 2 or 6 weeks of 1mg/kg 13-*cis*-RA treatment prior to behavioural testing, so it was therefore important to determine the retinoid plasma levels likely to be achieved through this drug treatment regime (see Chapter 2.2.3.). However, 13-*cis*-RA was not detected in a number of plasma samples of chronically treated rats. It is unlikely that the systemic level of 13-*cis*-RA reached, following chronic treatment, was below the threshold for detection and instead, it is likely to derive from the storage of plasma samples at -80°C for 6 months that may have resulted in the degradation of retinoids. Therefore, three adult rats were treated acutely with 1mg/kg 13-*cis*-RA (i.p., 1:1 DMSO:saline as per previous protocols), followed by blood collection and retinoid extraction described in Chapter 2.2.3. Results in Figure 3.15 show that all three rats achieve an average retinoid plasma concentration of 1.51 ± 0.05ug/ml (1.61, 1.45 and 1.47ug/ml for rats1-3 respectively). These findings are comparable with previous results from our group which demonstrated that in mice treated chronically with 1mg/kg 13-*cis*-RA, retinoid plasma levels were 1.5 ± 0.4ug/ml (O'Reilly *et al.*, 2006). Likewise, a previous study had shown that the plasma levels displayed in human patients administered 0.5mg/kg/day of 13-*cis*-RA was 0.74µg/ml (Kerr *et al.*, 1982).

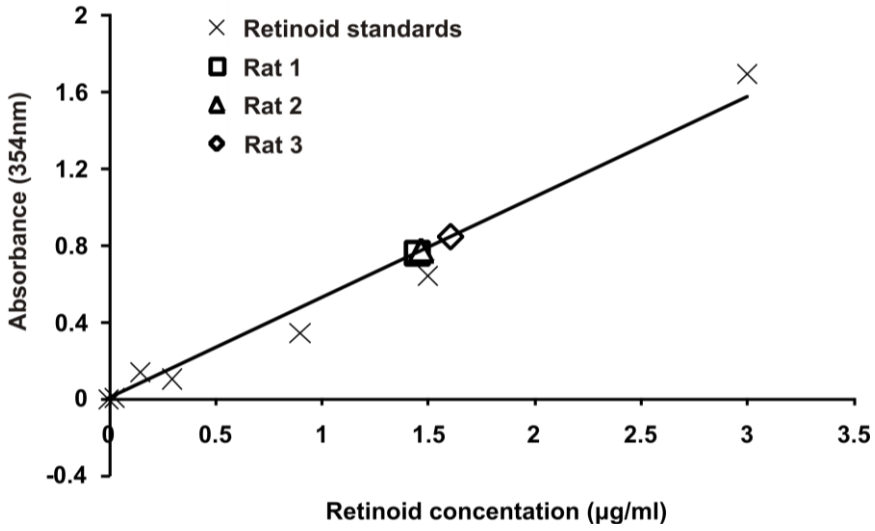

Figure 3.15: Retinoid extraction results from rats treated acutely with 13-*cis*-RA. A standard reference curve of plasma samples with known concentrations of 13-*cis*-RA was created by spectrophotometric analysis of samples at 354nm. Three rats were i.p. injected with 1mg/kg 13-*cis*-RA, followed by blood collection 30 min later. Retinoid extraction methods as previously described were carried out followed by spectrophotometric analysis of samples at 354nm.

3.4 Discussion

3.4.1 Discussion of all behavioural findings

The results presented in this chapter have shown that chronic administration of 1mg/kg 13-*cis*-RA has a significant effect on the behavioural profile of resident rats in the resident-intruder paradigm. The treatment of resident rats with 13-*cis*-RA (after 7 and 14 days) caused a significant reduction in aggressive behaviour towards intruder rats with a concomitant increase in submissive behaviours including flight submit and flight escape behaviour. This behavioural profile is therefore in direct contrast to the 'antidepressive' profile of chronic antidepressant treatment in the resident-intruder paradigm and is highly reminiscent to the behavioural profile of acute antidepressant treatment that may be associated with the worsening of depression symptoms in humans upon commencement of antidepressant treatment. The results obtained using chronic 13-*cis*-RA treatment are therefore suggestive of a pro-depressive effect.

Previous studies utilising the resident-intruder paradigm have shown that chronic treatment with antidepressants such as fluoxetine, paroxetine, venlafaxine, desipramine and many more, increase aggression and reduce flight-related behaviour of resident rats (Mitchell, 2005; Mitchell *et al.*, 2003; Mitchell *et al.*, 1992a; Mitchell *et al.*, 1997b). This behavioural profile is thought to reflect the increased levels of extrapunitive aggression (outwardly-directed and positive in nature) and assertiveness exhibited by human patients during their recovery from depressive illness through chronic antidepressant treatment (Priest *et al.*, 1980). Meanwhile, the acute antidepressant treatment of resident rats has been shown to reduce aggression and increase flight behaviour (Mitchell, 2005; Mitchell *et al.*, 1992a) that is thought to reflect decreased levels of assertiveness and a change towards intropunitive aggression (inwardly-directed, including feelings of guilt, suicide ideation etc) exhibited by human patients suffering with depression (Priest *et al.*, 1980). The resident-intruder paradigm therefore predicts the ability of antidepressant drugs to initially worsen the symptoms of depressive illness during the first few days of treatment; a controversial effect that appears to have been confirmed in some cases clinically (Cipriani *et al.*, 2005).

The increase in aggression and reduction in flight behaviour observed in resident rats following chronic antidepressant treatment are evident after 7 and 14 days of treatment and subsequently return to pre-treatment levels after 7 days of antidepressant cessation (Mitchell, 2005). Likewise, resident rats chronically treated with 13-*cis*-RA display an onset of reduced aggression and increased flight behaviour after 7 days 13-*cis*-RA administration and the behaviours were subsequently reversed to pre-treatment levels following one week cessation of drug treatment. The onset of pro-depressive behaviour in the resident-intruder paradigm (7 days) appears to differ from the onset seen in human patients treated with 13-*cis*-RA that typically ranges from a few days to months (reviewed by (Hull *et al.*, 2005)). However, one study suggests the median recovery time following 13-*cis*-RA de-challenge in human patients is 4.5 days (Wysowski *et al.*, 2001) and this closely reflects the results from the resident-intruder study, whereby discontinuation of 13-*cis*-RA treatment for one week was sufficient to reverse behaviour to pre-treatment levels. Clearly the ability of 13-*cis*-RA (and chronic antidepressant treatment) to alter resident rat behaviour is specific yet reversible upon discontinuation, that suggests that the underlying neuronal mechanisms responsible may be similarly specific yet reversible.

A number of studies have attempted to elucidate the underlying neuronal mechanisms responsible for increasing aggression and reducing flight behaviour following chronic antidepressant treatment of resident rats in the resident-intruder paradigm (Mitchell, 2005). They consist of temporal association studies between changes in aggression following venlafaxine, fluoxetine, paroxetine and electroconvulsive shock treatment and alterations in 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}R-mediated function (Mitchell *et al.*, 2003; Mitchell *et al.*, 2000a; Mitchell *et al.*, 1997b). The increased aggression following venlafaxine, fluoxetine and paroxetine treatment most closely followed a reduction in 5-HT_{2C}R-mediated function, as measured by hypolocomotion induced by acute challenge with the 5-HT_{2C}R agonist *m*-chlorophenylpiperazine (*m*CPP) (Mitchell *et al.*, 2000a). Although a reduction in 5-HT_{2C}R-mediated function was also observed after 3 days of electroconvulsive shock, an increase in 5-HT_{2A}R-mediated function was measured after 7 days of treatment that coincided exactly with increased aggression of resident rats (Mitchell *et al.*, 2003; Mitchell *et al.*, 2000a).

Overall, the suggestion is that altered 5-HT_{2C}R function may be responsible for antidepressant-induced behavioural alterations in the resident-intruder paradigm and may likewise be altered following 13-*cis*-RA treatment (discussed further in Chapter 6.3).

The FST was employed to test for depression-related behaviour, but was unable to demonstrate any effect of 1mg/kg/day 13-*cis*-RA treatment, after either 2 weeks or 6 weeks, in both adult and juvenile rats. The FST is the most widely used pharmacologic model for assessing acute antidepressant activity because of its ease of use, reliability across laboratories and ability to detect a broad spectrum of antidepressants (Cryan *et al.*, 2002). Conversely, increased immobility times in the FST have been measured to test the depressive effects of chronic amphetamine withdrawal (Cryan *et al.*, 2003), nicotine withdrawal (Zaniewska *et al.*, 2010), cocaine withdrawal and chronic mild stress (Frankowska *et al.*, 2009), olfactory bulbectomy (Tasset *et al.*, 2008) and social isolation (Ago *et al.*, 2008), that makes the FST a valid model for measuring the potential pro-depressive properties of 13-*cis*-RA. The lack of effect of 13-*cis*-RA treatment on adult rats tested in the FST is in agreement with previous studies that had treated adult rats with 7.5 and 22.5 mg/kg/day of 13-*cis*-RA for 12 to 19 weeks (Ferguson *et al.*, 2007b), as well as 7.5 and 30mg/kg/day of 13-*cis*-RA for 7 weeks (Ferguson *et al.*, 2005a) .

Our FST data also shows the lack of effect of 13-*cis*-RA treatment in juvenile rats, whereas previous work in our group had shown that juvenile mice treated with 1mg/kg/day 13-*cis*-RA for 6 weeks displayed increased immobility times with a concomitant decrease in swimming time that suggested a pro-depressive effect (O'Reilly *et al.*, 2006). Based on this data in juvenile mice, the original hypothesis was that juvenile animals would be more susceptible to the effects of 13-*cis*-RA. Unfortunately, it is not feasible to corroborate our findings of juvenile rats tested in the FST with the behaviour of juvenile rats in the resident–intruder paradigm test, given that juvenile animals do not engage in the same range of aggression behaviours that adult rats do. Juvenile rats engage in play fighting at about 18 days of age (pre-weaning) that peaks at about 30–36 days (Panksepp, 1981; Pellis *et al.*, 1992) and differs greatly from the adult aggression behaviour that is measured and validated in

the resident-intruder paradigm (Mitchell, 2005). Therefore, at present, our results suggest that while the FST is a sensitive model for revealing the pro-depressant effects of 13-*cis*-RA in juvenile mice, it is not for measuring juvenile rats. The reasons underlying these different effects in juvenile mice and juvenile rats are unlikely to derive from variations in the 13-*cis*-RA treatment regime employed, given that both groups of animals received i.p. injections of 1mg/kg/day 13-*cis*-RA (in 1:1 v/v DMSO:saline) for 6 weeks. One possibility for this apparent behavioural difference across the two species is that there is a species difference in the response to 13-*cis*-RA treatment. This could emanate from differences in the metabolism of 13-*cis*-RA administration or a difference in retinoid signalling pathways or non-retinoid-based differences in neurophysiology that could lead to diverging results in FST performance after treatment. A species difference between rat and mice sensitivity to retinoids is further supported by the observation that learning and memory are impaired following chronic 13-*cis*-RA treatment in mice (Crandall *et al.*, 2004) but not in rats (Ferguson *et al.*, 2007a).

There is also a discrepancy when comparing the pro-depressive effect of 13-*cis*-RA treatment of adult rats tested in the resident-intruder paradigm and the lack of effect in adult rats tested in the FST. This may be due to the resident-intruder model being more sensitive than the FST to the pro-depressant effects of 13-*cis*-RA, perhaps because the former model utilises social stress, which may be of greater relevance to depression pathology, whereas the latter is based on environmental stress. Similarly, the discrepancy found between the results of adult rats treated with 13-*cis*-RA tested in the resident-intruder and adult rats tested in the sucrose anhedonia paradigm, may derive from the different endophenotypes of depression that each test models. While the sucrose consumption test is able to model anhedonia, the inability to derive pleasure from pleasurable events, the resident-intruder paradigm is based on the increased flight behaviour (Dixon *et al.*, 1989), impaired sociability and increased intropunitive aggression (Priest *et al.*, 1980) displayed in depressed patients.

The sucrose consumption test, as a model of anhedonic behaviour, was used to test the pro-depressive effects of 1mg/kg/day of 13-*cis*-RA for 6 weeks, but found no effect after either 2 weeks or 6 weeks treatment, in both adult and juvenile rats. These results are in agreement with previous studies that

had shown the treatment of adult rats with 7.5 and 22.5mg/kg/day of 13-*cis*-RA for 2 to 16 weeks (Ferguson *et al.*, 2007b) and 7.5 and 30mg/kg/day 13-*cis*-RA for 3 to 10 weeks (Ferguson *et al.*, 2005a) was insufficient to alter sucrose solution consumption levels. A number of studies have validated the use of decreased sucrose consumption as a measure of anhedonia-related behaviour in rats following chronic mild stress (Papp *et al.*, 1991), whereas antidepressants, such as fluoxetine, are able to reverse the decrease in sucrose solution consumption, thereby implicating the involvement of serotonergic pathways in this model (Muscat *et al.*, 1992b; Willner, 1997). In fact, there is evidence that decreases in sucrose consumption induced by chronic mild stress can be reversed by injections of quinpirole and bromocriptine, both D2-like DA agonists and therefore implicates dopaminergic pathways in mediating the behavioural effects of this animal model (Muscat *et al.*, 1992a).

The open field test was utilised to test whether 13-*cis*-RA had any pronounced effects on locomotor behaviour (Walsh *et al.*, 1976). As mentioned previously, retinoid receptor knockout mice (RARβ-RXRβ$^{-/-}$, RARβ-RXRγ$^{-/-}$ and RXRβ-RXRγ$^{-/-}$) demonstrate deficits in total locomotion, rearings and fall latency as assessed by the open field and rotarod tests (Krezel *et al.*, 1998). The deficits in locomotor ability of these knockout mice were not thought to derive from muscle or peripheral nervous system deficiencies, but instead, due to the reduction of D1DRs and D2DRs in the ventral striatum (Krezel *et al.*, 1998). It is therefore thought that retinoid signalling is implicitly associated with locomotor regulation via altered signalling within the mesolimbic system. However, our studies show that treatment with 1mg/kg/day of 13-*cis*-RA for 2 and 6 weeks, in both adult and juvenile rats, did not alter the number of line crossings or vertical rears. These findings are similar to previous studies using adult rats treated with 7.5 and 22.5mg/kg/day of 13-*cis*-RA for 1 to 14 weeks (Ferguson *et al.*, 2005a) and juvenile mice treated with 1mg/kg/day of 13-*cis*-RA for 6 weeks (O'Reilly *et al.*, 2006). Therefore, excessive levels of retinoids do not appear to alter locomotion in juvenile or adult rats, in contrast to mice deficient in retinoid receptors. Furthermore, this finding suggests that the interpretation of FST data is unlikely to be obscured (given that the FST relies on detecting changes in immobility, swimming and climbing behaviour).

The chronic treatment of adult rats with 13-*cis*-RA did not affect 8-OH-DPAT-induced hypothermic responses, compared with vehicle-treated rats. 8-OH-DPAT is a 5-HT_{1A}R-selective agonist and is thought to induce hypothermic responses in rats via post-synaptic 5-HT_{1A}Rs (Bill *et al.*, 1991) and to some extent, 5-HT_7Rs in rats (Faure *et al.*, 2006; Hedlund *et al.*, 2004). We were confident that the hypothermic responses induced by 8-OH-DPAT were specific to 5-HT_{1A}Rs, given that the 5-HT_{1A}R specific antagonist WAY-100635 ablated hypothermic responses (Fletcher *et al.*, 1996; Forster *et al.*, 1995). Although 13-*cis*-RA treatment does not affect 5-HT_{1A}R-mediated hypothermia, it remains unclear if inferences can be made in regards to the function of post-synaptic 5-HT_{1A}Rs and their receptor numbers. One autoradiography study has shown that increased hypothermia induced by 8-OH-DPAT correlated with the increased binding of [3H]8-OH-DPAT to 5-HT_{1A}Rs that was therefore suggestive of an increase in 5-HT_{1A}R numbers (Knapp *et al.*, 1998). However, this relationship was only found in forebrain regions such as the frontal cortex and was not evident in the raphe and hypothalamus.

As a final observation, we recorded highly different behavioural performances between juvenile and adult rats in the FST, sucrose consumption paradigm and open field test, regardless of 13-*cis*-RA or vehicle treatment. Following two weeks of treatment, juvenile rats (Figure 3.7B) exhibited decreased time spent immobile compared to adult rats (Figure 3.7A) in the pre-swim test session of the FST (also shown in Figure 3.8). This effect was not observed in juvenile rats undergoing the second pre-swim test session (after 6 weeks of treatment), perhaps due to the maturation of juveniles into adults or factors associated with re-exposure to the test. We also observed increased sucrose solution consumption in juvenile rats compared to adult rats (Figure 3.10). The effect was irrespective of treatment, re-testing (2 and 6 weeks) and the length of observation required to measure consumption (1 h vs 2 h, Figure 3.10A and Figure 3.10B respectively). Furthermore, we recorded increased locomotor and exploratory behaviour in juvenile rats as measured by the open field compared with adult rats (Figure 3.11). Juveniles displayed an increased number of line crossings (Figure 3.11A) and vertical rears (Figure 3.11B) compared with adult rats, that was irrespective of treatment and evident upon the first (two weeks of treatment) and second (6 weeks of treatment) exposure to the test. In

addition to our observations, inherent differences in the behaviour of adult and juvenile animals have been widely reported in mouse anxiety-like behaviour (Slawecki, 2005) and depression-related behaviour (Hefner *et al.*, 2007), and support the idea that some aspects of human adolescence can be modelled in juvenile rodents that are 4–6 weeks of age (Spear, 2000).

All the behavioural models employed in this chapter are certainly highly validated models of antidepressant-like activity (Cryan *et al.*, 2002; Mitchell, 2005; Papp *et al.*, 1991), given their sensitivity to antidepressants such as TCAs and SRRIs. However, their specificity to model depression-related behaviour *per se*, is less clear as both TCAs and SSRIs are known to be equally efficacious at treating anxiety disorders in humans (Nutt, 2000), including panic disorders (Fahy *et al.*, 1992). This is perhaps not surprising given the comorbidity (and common symptoms) of depression and anxiety (Johnstone *et al.*, 1980; Wong *et al.*, 2001). One study was able to show that anxiolytics such as diazepam and alprazolam have no effects on FST immobility in unstressed animals, although the same study found that anxiolytics ablate the shortened immobility time of repeatedly cold-stressed animals (Hata *et al.*, 1995). Similarly others have reported that anxiolytics reduce immobility time when given in conjunction with behaviourally inactive doses of SSRIs (Da-Rocha *et al.*, 1997) and potentiate the reduction of immobility following TCA administration (Flugy *et al.*, 1992). Therefore, anxiolytics are capable of altering FST performance, although the sedative and/or motor effects of anxiolytics at higher doses may to confound the interpretations made. Similarly, benzodiazepines can heighten aggressive behaviours in resident rats of the resident-intruder paradigm (Gourley *et al.*, 2005), suggesting the model is also sensitive to anxiolytics, although the majority of drugs validated in this model, using rats, have been antidepressants (Mitchell, 2005). In contrast, anxiolytics are unable to reverse stress-induced anhedonia in animals, unlike antidepressants, suggesting this model is only sensitive to the effects of antidepressants (Muscat *et al.*, 1992b). Overall, these behavioural models were utilised as models of depression-related behaviour given their sensitivity to antidepressants (despite some sensitivity to anxiolytics), but more importantly, their ethological construct (stress, learned helplessness and social encounters) that differs considerably

from the one used in most anxiety based models (exploratory-based approach-avoidance) (Cryan *et al.*, 2005a).

3.4.2 Implications of resident-intruder findings and future work

Further evidence is required to substantiate the finding that 13-*cis*-RA induces a pro-depressive profile in the resident-intruder paradigm. Firstly, a resident-intruder study whereby the resident rats underwent chronic amphetamine withdrawal (Cryan *et al.*, 2003) or chronic mild stress (Willner, 1997), would be necessary to establish the profile of a depressive phenotype in the resident-intruder paradigm and allow subsequent comparison with the data we have obtained from 13-*cis*-RA-treated resident rats. Secondly, a protocol whereby resident rats received 13-*cis*-RA in parallel with antidepressant treatment would demonstrate whether the pro-depressive phenotype induced by 13-*cis*-RA can be ablated or reversed by antidepressants and perhaps suggest which monoaminergic pathways are involved.

The limitations and validity of the resident-intruder paradigm to accurately model depression-related behaviour must also be considered. For instance, the chronic treatment of healthy unmanipulated resident rats with antidepressants results in increased levels of aggression and reduced flight behaviour, whereas healthy, non-depressed people do not respond to antidepressant treatment. Furthermore, the specific effects of antidepressants on aggression levels may be limited to rats, given that no such association is present in resident-intruder studies using male mice (Lumley *et al.*, 2000). In fact, mouse resident-intruder studies have revealed the sensitivity of anxiolytics, rather than antidepressants, to altering aggression and further questions the validity of the resident-intruder to model depression-related behaviour via modified aggressive behaviour (Mitchell *et al.*, 2005). However, the species difference noted could simply reflect the inherent differences in aggressive behaviour between rats and mice, given that mice are violent when defending their territory whereas rats live in social groups and excessive violent behaviour can be detrimental.

The sensitivity of the resident-intruder paradigm to the behavioural effects of 13-*cis*-RA suggests that alternative social-based models, sensitive to the chronic effects of antidepressants, may be an important avenue of future research. Some of the other social-based models include neonatal and adult social isolation, social defeat and social hierarchy paradigms. Neonatal and adult social isolation has been shown to induce behaviour in non-human primates that is reminiscent of depressed and socially isolated children (Henn *et al.*, 1987; Robertson *et al.*, 1952). In one study, the impairment of social cooperation in isolated adult rats was reversed by chronic imipramine treatment and subsequently ablated by the 5-HT antagonist, metergoline (Willner *et al.*, 1989). The social defeat model analyses the defeat of one rodent by another during a social encounter and repeated defeat has been shown to be a form of chronic stress that is characterised by decreased aggressive behaviour (Albonetti *et al.*, 1994). Repeatedly defeated submissive C57BL/6J mice (by a dominant male mouse of the same strain), exhibited increases in immobility in the FST and this effect was reversed by chronic treatment with imipramine (Kudryatseva *et al.*, 1991). The social hierarchy paradigm is a closely related model and studies using this model have shown that the loss of the dominant status of a dominant rat within the group hierarchy is accompanied by decreased hedonia (determined by the abolition of morphine-induce place conditioning) and could be restored by chronic imipramine treatment (Coventry *et al.*, 1997; Willner *et al.*, 1995). Overall, these additional models lack the thorough validation of the resident-intruder paradigm but are potentially useful for confirming the behavioural effects of 13-*cis*-RA we have established in the resident-intruder paradigm.

In conclusion, our findings show that 13-*cis*-RA treatment alters resident rat behaviour aggression in the resident-intruder paradigm and may therefore reflect an increase in depression-related behaviour. Furthermore, previous studies have shown that serotonergic mechanisms underlie the alteration in resident rat behaviour. This would be in agreement with the hypothesis that 13-*cis*-RA treatment regulates monoaminergic molecular components via gene transcription, thereby causing pro-depressive behaviour.

Chapter 4

The gene and protein alterations mediated by 13-*cis*-RA administration *in vitro* and *in vivo*

4.1 Introduction

Our original hypothesis proposes that 13-*cis*-RA treatment, acting via retinoid receptors controlling gene transcription (see Figure 1.2), can alter the expression of genes thought to be involved in the pathology of depression (Chapter 1.4). The change in expression of depression-related genes would be reflected at the protein level and may result in functional changes (such as reduced serotonergic neurotransmission) that would contribute to the increased susceptibility to depression during 13-*cis*-RA treatment. Therefore, we have sought to elucidate the gene and protein components that may be altered by 13-*cis*-RA treatment both *in vitro* using a serotonergic neuronal cell line and *in vivo* using rat brain tissue. The rationale behind which neuronal genes were selected for analysis in this thesis was driven by evidence of *i)* links with depression pathology, *ii)* involvement in the action of antidepressants, *iii)* capability of regulating 5-HT neurotransmission and *iv)* known regulation by retinoids. I have therefore focused on the 5-HT_{1A}R, SERT, TPH2, 5-HT_{1B}R, MAOA, COMT and D2DR genes (described in detail in Chapter 1.3.1.2.-1.3.1.4.).

Briefly, 5-HT_{1A}R was chosen for analysis given that it has been associated with human depression and depression-related behaviour in animal models (Arango *et al.*, 2001; Heisler *et al.*, 1998). SERT is thought to be associated with the pathology of depression as it is the specific target of SSRIs, which are highly efficacious at treating depression (Backstrom *et al.*, 1989; Owens *et al.*, 1994), whereas autoradiography and single-photon emission-computed tomography studies suggest the density of SERT binding sites are altered (Malison *et al.*, 1998; Purselle *et al.*, 2003). Meanwhile, studies have shown that the expression of TPH2 is linked to changes in aggression, depression in humans and animals (Bach-Mizrachi *et al.*, 2006; Bach-Mizrachi *et al.*, 2008; Osipova *et al.*, 2009). The expression of 5-HT_{1B}Rs may be altered in depressed humans (Anisman *et al.*, 2008) and following antidepressant treatment in animals (Blier *et al.*, 1988). In fact, it is generally regarded that 5-HT_{1A}R (Hjorth *et al.*, 1991; Kreiss *et al.*, 1994), SERT (Blakely *et al.*, 1994; Invernizzi *et al.*, 1995; Lesch, 1997), TPH2 (Alenina *et al.*, 2009; Zhang *et al.*, 2004) and 5-HT_{1B}R (Sharp *et al.*, 1989; Starkey *et al.*, 1994) are all major regulators of 5-HT neurotransmission.

There is evidence for the involvement of D2-like receptors in depression in humans and animals (D'Haenen H *et al.*, 1994; Willner *et al.*, 1994) , but it is unclear whether D2DR is specifically involved. However, D2DR was selected for analysis in the study of 13-*cis*-RA-induced depression given that it is clearly regulated by retinoids (see Table 1.1. and Chapter 4.1.1.) and therefore provides a useful positive control. The enzyme MAOA was selected for analysis as it has a higher affinity for 5-HT compared with MAOB (Nagatsu, 2004) and is therefore viewed as the principal enzyme of 5-HT degradation. MAOA levels may be increased in depressed subjects (Meyer *et al.*, 2006) whereas MAOIs are efficacious at treating depression (Riederer *et al.*, 2004). COMT metabolises DA/NA (Alexrod *et al.*, 1958) and there is evidence of altered COMT activity in depressed humans (Shulman *et al.*, 1978), COMT polymorphisms associated with depression treatment (Benedetti *et al.*, 2009; Domschke *et al.*, 2009) and a reduction in depression-related behaviour in animals treated with a COMT inhibitor (Moreau *et al.*, 1994).

4.1.1. Potential regulation of monoaminergic components by retinoids

Given the strong evidence of an association between the 5-HT_{1A}R and depression pathology, the findings by Charest *et al.* that retinoids are able to regulate the expression of 5-HT_{1A}R *in vitro* putatively implicates retinoids with depression pathology (Charest *et al.*, 1993). A hybrid murine cell line, SN-48, was created by fusing 21 day postnatal mouse septal neurons with a murine neuroblastoma (Lee *et al.*, 1990). The treatment of the SN-48 cell line with ATRA (10µM, 24-98 h) resulted in the presence of 5-HT_{1A}R mRNA in differentiated SN-48 cells that was previously undetected in non-differentiated SN-48 cells. There is therefore a strong possibility that 13-*cis*-RA, like ATRA, may regulate 5-HT_{1A}R gene transcription.

Meanwhile, a study using the rat pituitary cell line MMQ (Judd *et al.*, 1988) has shown that ATRA treatment (1 h, 1µM) induces a two-fold increase in D2DR mRNA, while 48 h of treatment induced a 30-fold increase in D2DR mRNA (Samad *et al.*, 1997). The same study was able to show that the D2DR promoter had a verified RARE in MMQ cells and RXR$\gamma^{-/-}$ mice had a 40% reduction of D2DR

mRNA in the striatum compared with wildtypes. Similarly, RARα-RXRγ$^{-/-}$ and RXRγ–RXRβ$^{-/-}$ mutant mice displayed a 60% reduction of D2DR mRNA in the striatum compared with wildtypes, while RARβ–RXRγ$^{-/-}$ mice had a 70% reduction. Other studies have demonstrated the regulation of D2DR by retinoids including an increase in D2DR mRNA in primary striatal cells following ATRA treatment (Valdenaire *et al.*, 1998) and an increase in D2DR mRNA expression and function in human teratocarcinoma NT2 cells following ATRA treatment (Sodja *et al.*, 2002). These studies implicate retinoids in the regulation of D2DR gene expression.

It is currently unclear whether SERT, TPH2, 5-HT$_{1B}$R, MAOA and COMT gene transcription are under the regulation of retinoids such as ATRA and 13-*cis*-RA both *in vitro* or *in vivo* (for review see (Lane *et al.*, 2005)). We are unaware of any promoter studies conducted in the promoter of these genes to determine the presence of a RARE or other studies suggesting ATRA can upregulate/downregulate mRNA expression and likewise, alter protein levels. However, the large body of evidence that links these monoaminergic genes with depression pathology means they are good candidates for investigating the molecular mechanisms of 13-*cis*-RA-induced depression.

In the present study, the raphe nuclei and the hippocampus were selected for *in vivo* gene expression analysis due to the evidence that implicates these brain regions with the neuropathology of depression. As already mentioned in Chapter 1.3.1.2., serotonergic neurons derive from the raphe nuclei and contain large numbers of pre-synaptic 5-HT$_{1A}$Rs, SERT and to a lesser extent 5-HT$_{1B}$Rs, along with high concentrations of TPH2 enzyme and to a lesser extent, the MAOA enzyme. Meanwhile, the hippocampus is also thought to be involved in the neuropathology of depression given that it receives serotonergic inputs from the raphe nucleus and contains a high concentration of post-synaptic 5-HT$_{1A}$Rs (Lesch *et al.*, 2004; Sharp *et al.*, 2007). Post-mortem studies have shown that depressed suicide victims have reduced numbers of 5-HT$_{1A}$Rs in the hippocampus ((Cheetham *et al.*, 1990) and reviewed in (Savitz *et al.*, 2009)), while there have been consistent reports of hippocampal atrophy in depressed patients (Bremner *et al.*, 2000; Sheline *et al.*, 1996). Furthermore, rats undergoing the chronic mild stress paradigm had reductions in post-synaptic 5-HT$_{1A}$R mRNA expression and ligand

binding in the hippocampus and the effect could be reversed by imipramine treatment (Lopez *et al.*, 1998).

For the *in vitro* investigation of gene expression following retinoid treatment, we utilised the rat raphe nuclei RN46A-B14 cell-line. This cell line has been shown to differentiate towards a serotonergic-like phenotype as demonstrated by 5-HT synthesis and release (White *et al.*, 1994), 5-HT_{1A}R binding (Eaton *et al.*, 1995) and a high expression of 5-HT_{1B} receptors (Rumajogee *et al.*, 2006). Therefore, both the raphe (from rat tissue and the RN46A-B14 cell line) and the hippocampus are appropriate brain regions for analysing 13-*cis*-RA-induced gene changes that may underlie alterations in behaviour.

In the present study we sought to determine the chronic effects of 13-*cis*-RA treatment on gene and protein levels *in vivo* using adult and juvenile rats and *in vitro*, using the RN46A-B14 raphe nuclei cell line. The neuronal genes of interest were 5-HT_{1A}R, SERT, TPH2, 5-HT_{1B}R, MAOA, COMT and D2DR, and were quantified using quantitative real-time RT-PCR. Additionally, for all gene expression experiments, we analysed the gene expression of the retinoid receptors RARα and RARβ. This acted as a positive control given that they both contain RAREs in their respective promoter regions and retinoids induce their expression (Brand *et al.*, 1988; Lane *et al.*, 2005; Petkovich *et al.*, 1987) We further investigated the effect of 13-*cis*-RA treatment by selectively analysing protein level changes in certain genes of interest in the RN46A-B14 cell line and adult/juvenile raphe nuclei and hippocampal tissue using semi-quantitative Western blotting.

4.2 Methods

4.2.1 Animals

Male Wistar rats (Charles River, UK) were treated for a period of 6 weeks daily with either vehicle (1ml/kg, saline:DMSO 1:1 ratio) or 13-*cis*-RA (1mg/kg in vehicle, described in section 2.2.1.). Adult rats were 8 weeks of age upon commencement (270-305g), while juvenile animals were 4 wks old at the start of treatment (75-100g) which corresponds to a time of sexual immaturity and brain remodelling analogous to human adolescence (Spear, 2000). The raphe, hippocampus and prefrontal cortex of untreated control rats (adult Wistar rats, University of Bath, 250g-350g) and treated rats were microdissected for gene expression studies (see Chapter 2.2.2.).

4.2.2. RNA isolation

TRIzol reagent (Invitrogen) was used according to the manufacturer's protocol for the isolation of total RNA from rat brain tissue and RN46A-B14 cells. Briefly, tissue was homogenized by adding 0.5ml of TRIzol reagent and mixed with a pellet pestle (Sigma). A further 0.5ml of TRIzol was added and the homogenate was passed through a 23G needle (BD Microlance, Fisher). Homogenized samples were then left to stand for 5 min at room temperature, before 200µl of chloroform was then added. Tubes were shaken vigorously for 15 s and then left to incubate at room temperature for 2 min. The samples were subsequently centrifuged at 13,000rpm for 15 min at 4°C (all centrifugation steps were at 13,000 rpm at 4°C unless otherwise stated) and the upper aqueous phase was kept. The RNA was precipitated by adding 0.5ml of propan-2-ol and incubated at room temperature for 10 min. After centrifugation for 10 min, the supernatant was removed and 1ml of 75% ethanol (Fisher) was added to wash the RNA pellet. Eppendorfs were centrifuged at 8000 rpm for 5 min and the liquid was removed, leaving the RNA pellet to air dry.

The air-dried RNA pellet was resuspended in 30µl of RNAse-free water. To remove DNA contamination of RNA, a DNAse digest was carried out: 30µl RNA, 4µl NEB buffer (New England Biolabs), 1µl RNAsin (Fermentas), 2µl DNAse (10U/µl, Roche) and 3µl RNA-free water in a water

bath at 37°C for 30 min. The RNA was then reprecipitated by adding 80µl 100% ethanol and left for 10 min at room temperature. The eppendorf tubes were centrifuged for 15 min and the supernatant was removed. The pellet was washed with 250µl of 75% ethanol and vortexed to resuspend the pellet. The tubes were centrifuged for 5 min, followed by removal of the supernatant and the remaining pellet was left to air dry. Once dry, the pellet was resuspended in 20µl of RNAse-free water and stored at -80°C.

RN46A-B14 cells underwent a similar RNA isolation protocol. After 48 h of retinoid treatment (described in Chapter 2.3.1.), differentiation media was removed from the 6-well plate and the cells were rinsed with sterile phosphate buffered saline (PBS) solution. PBS (0.1M, pH 7.4) was made by dissolving 2.7g sodium phosphate monobasic (NaH_2PO_4, Acros Organics), 11.5g sodium phosphate dibasic (Na_2HPO_4, Fisher) and 9g sodium chloride (NaCl) into milliQ water (total of 1L volume). Following aspiration of PBS, 1ml of TRIzol was added and cells were homogenised with a cell scraper. Cells were transferred to eppendorf tube using a 23G needle and 200µl of chloroform was added and subsequent steps were followed as per the protocol above.

The concentration of isolated RNA from both tissue and cells was confirmed using spectrophotometric methods to measure absorbance at 260nm and determined using A_{260} =1 for 40µg/ml solution. Meanwhile, the purity was assessed via 260nm/280nm absorbance ratios.

4.2.3. One-step reverse transcription PCR (RT-PCR)

The presence of our genes of interest (GOI) in both the RN46A-B14 cell line and untreated adult rat (raphe nuclei, hippocampus and prefrontal cortex) was first confirmed using one-step RT-PCR (Invitrogen) with the gene specific primers (Invitrogen) shown in Table 4.1. A variety of SERT (1-3) and TPH2 (1-2) primers were tested and SERT (1, 2) and TPH2 (1, 2) were used for one-step RT-PCR. One-step RT-PCR reactions were performed using Superscript™ One-Step RT-PCR with Platinum® *Taq* (Invitrogen). Master mixes were created on ice and the quantities for each PCR

Primer	Sequence	Amplicon length (base-pairs)	References
SERT (1)	FOR: TTTGCCATCATCTTCTTCCTCATG REV: GGCCACCCAGCAGATCCTC	Rat (359)	(Filipenko *et al.*, 2002)
SERT (2)	FOR: CGTCATCTGCATCCCTACCTATAT REV: TCTGTGGGTGTTTCAGGAGTGATAC	Rat (101)	(Koulmann *et al.*, 2006)
SERT (3)	FOR: ACTGGGCCAGTACCACCG REV: TCGGGCAGATCTTCCTCC	Rat (21)	(Suda *et al.*, 2008)
5-HT$_{1A}$R	FOR: CCGCACGCTTCCGAATCC REV: TGTCCGTTCAGGCTCTTCTTG	Rat (108)	(Kindlundh-Hogberg *et al.*, 2006)
5-HT$_{1B}$R	FOR: CACCCTTCTTCTGGCGTCAAG REV: ACCGTGGAGTAGACCGTGTAG	Rat (93)	(Kindlundh-Hogberg *et al.*, 2006)
TPH2 (1)	FOR: GGTTCCCTCGGAAGATCTGAG REV: CAGAGCTCCCGGAACACAAC	Mouse (224)	(Matsuda *et al.*, 2004)
TPH2 (2)	FOR: TAAATACTGGGCCAGGAGAGG REV: GAAGTGTCTTTGCCGCTTCTC	Rat (132)	(Sugden, 2003)
D2DR	FOR: TCGCCATTGTCTGGGTCCTGT REV: TGCCCTTGAGTGGTGTCTTCA	Rat (255)	(Viyoch *et al.*, 2001)
MAOA	FOR:CAAGCAAGACACGCTCAGGAA REV: ATACGCAAATTCCCGAGCAGT	Rat (92)	(Lindley *et al.*, 2005)
COMT	FOR: CACCTACTGCACACAGAAGGAA REV: AGTAGCCACAGTAAGCTCCCAGT	Rat (124)	(Lindley *et al.*, 2005)
RARα	FOR: CTGGAGATGGACGATGCTGAGACT REV: CACAGATGAGGCAGATGGCACTGA	Rat (54)	(Bry *et al.*, 2006; O'Reilly *et al.*, 2007)
RARβ	FOR: CAAAGCCTGCCTCAGTGGATTCA REV: AGTGGTAGCCCGATGACTTGTCCT	Rat (178)	(Bry *et al.*, 2006; O'Reilly *et al.*, 2007)

RARγ	FOR: GGAACTCATCACCAAGGTCAGCAA REV: CGCTTCGCAAACTCCACAATCTT	Rat (175)	(Bry *et al.*, 2006; O'Reilly *et al.*, 2007)
RXRα	FOR: CTTTGACAGGGTGCTAACAGAGC REV: ACGCTTCTAGTGACGCATACACC	Rat (172)	(Nishizawa *et al.*, 2003)
RXRβ/γ	FOR: AGGCAGGTTTGCCAAGCTTCTG REV: GGAGTGTCTCCAATGAGCTTGA	Rat (102)	(Husson *et al.*, 2003)
rRNA	FOR: GTAACCCGTTGAACCCCATT REV: CCATCCAATCGGTAGTAGCG	Human (114)	(Schmittgen *et al.*, 2000)
β-actin	FOR:ACCAACTGGGACGATATGGAGAAGA REV:TACGACCAGAGGCATACAGGGACAA	Mouse (166)	(Schmittgen *et al.*, 2000)

Table 4.1: Gene-specific forward and reverse primers for GOI. Forward and reverse primers sequences were derived from published sequences and sequences underwent Basic Local Alignment Search Tool (BLAST) analysis to confirm the presence of the correct amplicon and expected amplicon size (in base pairs). Primers were used for both one-step RT-PCR and real-time RT-PCR.

reaction were as follows: 12.5µl 2X Reaction Mix containing 0.4mM of each dNTP, 24mM $MgSO_4$, 10.1µl RNA-free water, 0.4µl of RT/Platinum *Taq* Mix and 1µl template RNA (0.25µg/µl). Forward and reverse primers (0.5µl at 25µM) were pipetted into 0.2ml PCR tubes, before 23µl of the master mix was also added. PCR tubes were vortexed and centrifuged (5,000 rpm, 1 min, room temp.), before being placed in the PCR machine (DNA Engine Peltier Thermal Cycler , PTC-200, MJ Research). Positive controls were created by amplifying the housekeeper genes β-actin and/or ribosomal RNA (rRNA), while the negative controls were created by the omission of the 30 min cDNA synthesis step (placed on ice during this time). RNAse free reagents, plastics and filter pipette tips were used at all times to prevent cross-contamination.

Conditions for one step reverse transcription PCR amplification were as follows: a cDNA synthesis step at 50°C for 30 min followed by 94°C for 2 min, before denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for a total of 40 cycles. A final extension step at 72°C for 5 min took place. PCR products were then electrophoresed on a 1.2% agarose gel (70 min at 95mV). Gel pictures were captured using GeneSnap (SynGene, 3.00.15) software.

4.2.4. Quantitative real-time RT-PCR

To quantitatively measure the expression of our GOI in rats and cell lines, quantitative real-time RT-PCR was used in a two step process. Firstly, template RNA was reverse transcribed into cDNA using the Omniscript RT kit (Qiagen). This was achieved by adding 2.5µl of 10X RT buffer (Qiagen), dNTP mix (Qiagen) and random primers (Invitrogen), along with 1.5µl of RNAsin (10U/µl), 1µl RTase (Qiagen) and 11 µl of RNA-free water to 1.5µl of RNA (0.5µg/ µl) in PCR tubes. The resulting mixture (22.5µl containing 33.3ng of RNA template) was vortexed and centrifuged, before tubes were incubated at 37°C for 1 h to create cDNA template. The concentration of RNA template and therefore cDNA, were optimised in subsequent experiments and are noted in future chapter sections.

Secondly, fresh cDNAs (1.25µl) were added to a reaction mix containing GOI-specific forward and reverse primers (0.4µl at 25µM), 6.95µl RNA-free water and 1µl LightCycler FastStart DNA Master ^{PLUS} SYBR Green I (Roche). The primers used were the same as shown in Table 4.1.The concentration of the primers was subsequently optimised according to the GOI and source of RNA used. Reaction mixes were transferred to LightCycler glass capillaries (Roche), along with a complete repeat of all mixtures to create to two technical repeats. To control against non-specific amplification, a ‘no template control’ was created by the absence of cDNA template in the reaction mixture. All mixtures were spun at 4000rpm for 20 s at 4°C, before insertion into the LightCycler 2.0 Instrument (Roche).

The real-time RT-PCR amplification conditions for 5-HT_{1A}R, 5-HT_{1B}R, DDR2, COMT, MAOA, RARα, RARβ and rRNA were: 50°C for 2 min and 95°C for 2 min (both with 20°C/s ramp rate), denaturation at 95°C for 5 s, annealing at 62°C for 10 s and extension at 72°C for 15 s (all with 20°C/s ramp rate) for 40 cycles. Amplification conditions for SERT (Suda *et al.*, 2008) were identical with the exception of an annealing temperature of 60°C. Meanwhile, conditions for TPH2 (Sugden, 2003) was: 50°C for 2 min and 95°C for 2 min (both with 20°C/s ramp rate), denaturation at 95°C for 15 s, annealing at 57°C for 20 s and extension at 72°C for 10 s (all with 20°C/s ramp rate) for 40 cycles.

Conditions for all melting curve analyses were 95°C for 0 s (20°C/s ramp rate), 65°C for 15 s (20°C/s ramp rate) and 95°C for 0 s (0.1°C/s ramp rate), while the final step was 37°C for 10 s (20°C/s ramp rate). Melting curve and melting peak analysis (Tm) was performed using LightCycler Software 4.0. to indicate the specificity of the primers and the amplicons produced. Single product amplicons create single, clean peaks in melting curve analysis whereas contamination, mispriming and primer-dimers create small secondary peaks.

Optimal amplification of GOI amplicons was largely found by varying the annealing temperature and duration of the annealing step. To achieve this, one-step RT-PCR reactions were run on a temperature gradient with an initial denaturation step of 95°C for 15 min, and then 40 cycles of 94°C for 15 s, 64°C for 30 s (at a gradient of 12°C) and 72°C for 1 min. Final extension was for 10 min at 72°C. RT-PCR products were then run on a gel as previously described and greater band intensity signified optimal conditions.

4.2.4.1. Comparative threshold cycle method ($2^{-\Delta\Delta Ct}$)

The exponential amplification of PCR products, known as the crossing point or threshold cycle (C_T) number, was automatically calculated using LightCycler Software 4.0. Gene changes were quantified using the comparative threshold cycle method ($2^{-\Delta\Delta Ct}$). Firstly, ΔC_T is calculated by normalizing the

threshold cycle number of the gene of interest to the housekeeping gene rRNA. The difference between the averaged ΔC_T of vehicle- treated cells/tissue and the averaged ΔC_T of 13-*cis*-RA-treated cells/tissue gives $\Delta\Delta C_T$ and is subsequently transformed to the equation $2^{-\Delta\Delta Ct}$ (Schmittgen *et al.*, 2000). The averages of all four sets of data required in this comparison are used and the overall standard deviation is calculated (total standard error= $\sqrt{}$ [(standard deviation 1)^2 + (standard deviation 2)^2 +...]). This simply calculates the standard deviation for $\Delta\Delta Ct$ and so the standard deviation is therefore exponentiated to the base two, to obtain the standard deviation for $2^{-\Delta\Delta Ct}$.

4.2.5. DNA sequence analysis

To confirm the specificity of the real-time RT-PCR amplicons, amplicons were extracted from gels and sent for DNA sequence analysis (Geneservice). Firstly, one-step RT-PCR was performed using untreated rat raphe RNA template with PCR conditions that were equivalent to that of the real-time RT-PCR protocol described above. Similarly, the primers used were identical to those used in real-time RT-PCR (rRNA, TPH2 (2), SERT (3), 5-HT_{1A}R and D2DR). The amplicons were separated on an agarose gel and visualised using an UV transilluminator. The appropriate DNA fragments were excised with a scalpel and extraction of the DNA fragment from the gel was carried out as per the manufacturer's instructions (QIAquick gel extraction, Qiagen). The subsequent PCR products were then sent for DNA sequence analysis (Geneservice).

4.2.6. Semi-quantitative Western blotting

Western blotting is a process that enables identification and quantification of specific proteins through a three-step process: separation of proteins by size via gel electrophoresis, the transfer of proteins to a polyvinylidene fluoride membrane and lastly probing of the membrane with protein-specific antibodies.

4.2.6.1. Sample preparation

Samples of brain tissue (stored at -80°C) were homogenised in 10 volumes of Radio Immuno Precipitation Assay buffer (RIPA buffer, as per Abcam protocol) to allow for protein release and solubilisation. RIPA buffer was made by adding 870mg of NaCl (150mM), 1ml of Triton X-100 (1%), 500 mg of sodium deoxycholate (0.5%), 100mg of sodium dodecyl sulphate (SDS, 0.1%) and 5ml of TRIS stock solution (50mM,pH 7.4) to milliQ water made up to a volume of 100ml. A 12.5X stock of RIPA buffer with protease inhibitors was created by adding one protease inhibitor cocktail tablet (Roche) to 2ml of RIPA and was subsequently added to the appropriate volume of RIPA buffer (eg. 0.8ml to 10ml). The addition of protease and phosphatase inhibitors to RIPA buffer slows down proteolysis, dephosphorylation and denaturation. The solution was spun at 1000 rpm for 3 min at 4°C and supernatant taken.

RN46A-B14 and MDA-MB-468 (negative control) cell lysates were prepared using a different process. Following 48 h of 13-*cis*-RA treatment, differentiation media was removed from the 6-well plates and the cells were washed with 1ml/well of ice-cold PBS. Following aspiration, 1ml/well of ice-cold RIPA lysis buffer (with protease inhibitors) was added and adherent cells were scraped off the wells with a cold plastic cell scraper. Cell lysates were triturated with a syringe needle (23G, Microlance) and transferred to microcentrifuge tubes on ice. The cell suspension was constantly agitated with a rocker for 30 min at 4°C, followed by microcentrifugation for 20 min at 12,000 rpm at 4°C and supernatant was kept for protein estimation.

4.2.6.2. Protein estimation

Protein estimation was carried out using the bicinchinic acid (BCA) Protein Assay kit (Pierce), whereby a series of protein standards were made by serial dilution with stock bovine serum albumin (BSA, 2mg/ml, Pierce) as summarised in Table 4.2. 50µl of known BSA protein standards, along with 50µl of diluted tissue or cell culture lysate and 50µl of milliQ water (acting as a blank), were pippetted into eppendorf tubes. To each of the tubes, 1ml of working reagent (50 parts BCA reagent A

Volume of BSA	Volume of diluent (milliQ water)	Final BSA concentration
100ul (stock)	700ul	250ug/ml (A)
400ul (A)	400ul	125ug/ml (B)
300ul (B)	450ul	50ug/ml (C)
400ul (C)	400ul	25ug/ml (D)
100ul (D)	400ul	5ug/ml (E)

Table 4.2: Dilution series of BSA protein standards (A-E) for Western blotting analysis. BSA standards with known protein concentrations were created by a series dilution of stock BSA (2mg/ml, Pierce) with milliQ water.

with 1 part BCA reagent B, Pierce) was added and mixed well. All standards and samples were incubated at 60°C for 30 min and immediately kept at 4°C. The standards, samples and blank were then measured with a spectrophotometer at 562nm (zeroed using water). The absorbance at 562nm for the blank was then subtracted from the absorbance readings of the standards and unknown samples. A standard curve was plotted using the absorbance readings for each BSA standard against its concentration in µg/ml and then used to calculate the unknown concentration of samples.

4.2.6.3. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transfer

The process of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) enables the separation of denatured proteins by the length/size of polypeptide chains. Firstly, two glass plates were cleaned with 70% ethanol, before being inserted into a casting frame and the frames were held by a casting stand (Bio-rad). A 10% separating gel was pipetted between the glass plates and was created using 2ml of 30% acrylamide (58.4g of acrylamide and 1.6g bis-acrylamide in 200ml milliQ water), 1.5ml of 4X separating buffer (36.34g of Tris base, 1.5M, and 0.8g SDS, 0.4%, in 200ml milliQ water), 2.5ml of milliQ water, 20µl of 10% w/v ammonium persulfate (APS, made fresh each time, Fluka) and lastly, 8µl of tetramethylethylenediamine (TEMED, Fluka) to begin polymerisation of the gel. The gel was covered by cold pronan-2-ol (to create an even top surface) and the gel was left to polymerise for 60 min at room temperature.

After the removal of pronan-2-ol, a 3% stacking gel was loaded above the separating gel. The stacking gel was made using 0.65ml of 30% acrylamide, 1.25ml of 4X stacking buffer (12.11g Tris base, 0.5M, and 0.8g SDS, 0.4%, in 200ml milliQ water), 3ml of milliQ water, 25µl of APS and 5µl of TEMED. Wells for loading of samples were created by insertion of a 10 lane (2mm) comb into the stacking gel and the gel was left to set for 30min. Once set, the gel plate sandwich was placed in a clamping frame and electrode assembly within a tank containing 1X SDS running buffer. 10X SDS running buffer solution was made using 30.2g Tris base, 144g glycine and 10g SDS in 1L of milliQ water.

Protein samples were denatured through the addition of an anionic denaturing detergent (SDS) and by boiling the mixture. Firstly, sample buffer was added to each protein sample: 6X sample buffer was created using 7ml of stacking buffer, 3.8g glycerol (~3ml), 1g sodium dodecyl sulphate, 0.93g of dithiothreitol (stored at 4°C), 1.2mg bromophenol blue and brought to a volume of 10ml with milliQ water. The sample buffer/protein mixture was then heated at 95°C for 5 min and then pulsed at 4°C. Subsequently, 10µl of each protein sample was loaded in each well of the gel, with the total amount of protein in each lane ranging from 7.5-40µg (depending on which tissue region or cell line was used). Additionally, 5µl of a protein ladder was loaded onto the gel (precision plus protein standard, Bio-rad). The samples were subsequently electrophoresed until they had migrated towards the bottom of the gel (80mA for 60 min).

Proteins on the gel were transferred to a polyvinylidene fluoride (PVDF, Bio-rad) membrane pre-incubated in 100% methanol. Firstly, a gel/membrane sandwich was created in a gel holder cassette in the following order from cathode to anode: wetted fibre pad/wetted filter paper/gel/membrane/wetted filter paper/wetted fibre pad. The gel holder cassette was placed within an electrode assembly in a tank containing 1X transfer buffer (22.5g glycine, 4.8g Tris base and 400ml methanol in 2L of milliQ water). Application of an electrical current (14V, overnight, 4°C) allowed for the electrophoretic transfer of protein from the gel to the membrane.

4.2.6.4. Immunolabelling

All blots were immunolabelled with either primary TPH2 (rabbit polyclonal, PA1-778, Cambridge Bioscience, ABR), 5-HT_{1A}R (rabbit polyclonal, ab64994, Abcam), SERT (rabbit polyclonal, AB10514P, Millipore) and D2DR (rabbit polyclonal, ab21218, Abcam) antibodies, followed by the re-probing of all blots with the loading control β-actin antibody (rabbit polyclonal, ab8227, Abcam). Validation of the specificity of immunolabelling came from observing a single band that was of the correct size (TPH2: 56kDa, 5-HT_{1A}R: 46kDa, SERT: 70kDa, D2DR: 58/61kDa and β-actin: 47kDa). Blots for TPH2, 5-HT_{1A}R, SERT and β-actin were blocked in non-fat dried milk (5% w/v, Bio-rad)/Tween Tris buffered saline (TBST) solution at room temperature for 1 h, while immunolabelling for D2DR required 2 h of incubation. TBST (pH7.4) was made by adding 50ml Tris stock solution (0.05M), 8.766g NaCl and 0.1% Tween 20 in 1L of milliQ water.

Rabbit anti-5-HT_{1A}R (1:300 in 2% milk/TBST) and rabbit anti-β-actin (1:2,500 in 2% milk/TBST) were incubated with blots for 1 h at room temperature, meanwhile rabbit anti-TPH2 (1:800 in 2% milk/TBST), rabbit anti-SERT (1:300 in 5% BSA/TBST) and rabbit anti-D2DR (1:300 in 5% milk/TBST) were incubated with blots at 4°C overnight.

Blots labelled with primary antibodies anti-TPH2, anti-5-HT_{1A}R, anti-D2DR and anti-β-actin were all subsequently incubated with a peroxidise-conjugated secondary goat anti-rabbit antibody (Chemicon, AP132P) diluted in 2% milk/TBST (1:2,000, 1:4,000, 1:5,000 and 1:8,000 respectively) for 1 h at room temperature. Meanwhile, blots incubated with primary anti-SERT were incubated with a peroxidise-conjugated secondary donkey anti-rabbit antibody (Millipore, AP182P) in 3%milk/TBST (1:5,000) for 1 h at room temperature. Blots were washed 4 times (7 min each) with fresh TBST solution after blocking, primary antibody and secondary antibody incubations.

Each blot was then incubated in enhanced chemiluminescence solution (1:1 ratio of detection reagents 1 and 2, Pierce) for 1 min at room temperature and exposed to X-ray film (Fuji film) in a film cassette

(hypercassette, Amersham). After the required time had elapsed, the X-ray film was transferred to a developer (Fuji x-ray film processor, Fuji) and the bands on the X-ray film were quantified using densitometric analysis software (Lab Image 2.7.2). Bands of the protein of interest were calculated relative to the loading control β-actin and averaged over two technical repeats. Values were subsequently averaged across biological repeats (n=3-4) in both vehicle-treated and 13-*cis*-RA-treated groups and protein levels of 13-*cis*-RA-treated samples were normalized to protein levels in vehicle-treated samples.

The subsequent re-probing of blots required the stripping of antibodies bound to the blots. This was achieved by two 10 min incubations with stripping buffer (15g glycine, 1g SDS, 10ml Tween 20 made up to 1L, pH2.2), followed by two 10 min washes with PBS.

4.3 Results

4.3.1. The expression profile of GOI *in vivo* and *in vitro*

One-step RT-PCR was employed to qualitatively establish the presence or absence of the GOI in the *in vitro* model (RN46A-B14 cells) and the rat raphe nuclei, prefrontal cortex and hippocampus tissue. The GOI analysed were the retinoid receptors RARα, RARβ, RARγ, RXRα and RXRβ/γ genes and the monoaminergic-related genes TPH2, SERT, 5-HT_{1A}R, 5-HT_{1B}R, D2DR, MAOA and COMT, while rRNA was used as a positive control. The expression profile of RARα, RARβ, RARγ, RXRα and RXRβ/γ genes *in vivo* and *in vitro* are shown in Figure 4.1. The results show particularly high levels of RARα, RARβ and RXRα gene expression in the rat raphe nuclei tissue with a marginally lower expression of RARγ and RXRβ/γ genes. This is a novel finding, as it has only been previously reported that CRABP I mRNA expression is expressed in the raphe nuclei of adult mice (Zetterstrom *et al.*, 1999). The presence of retinoid-signalling 'machinery' in the rat raphe nuclei suggests that it is the site of inherent retinoid signalling whereby the transcription of neuronal genes in this brain region may be regulated via RAR/RXR interactions. Similar to the rat raphe nuclei, all RAR and RXR genes were expressed in the RN46A-B14 cell line, which confirms its suitability as an *in vitro* model of retinoid-signalling in the raphe nuclei.

Meanwhile, Figure 4.2 shows the gene expression of TPH2, SERT, 5-HT_{1A}R and 5-HT_{1B}R in both RN46A-B14 cells and the rat raphe nuclei tissue. Qualitative analysis of TPH2, SERT, 5-HT_{1A}R and 5-HT_{1B}R gene expression via one-step RT-PCR reveals that all of these genes are expressed in the rat raphe nuclei tissue and in the RN46A-B14 cell line. However, both SERT and TPH2 do not appear to be expressed to the same degree in the cell line, compared with raphe tissue. However, previous studies have shown that the RN46A-B14 cell line does express TPH2 (White *et al.*, 1994) and SERT (Koldzic-Zivanovic *et al.*, 2006), along with 5-HT_{1A}Rs (Koldzic-Zivanovic *et al.*, 2006; Rumajogee *et al.*, 2006) and 5-HT_{1B}Rs (Koldzic-Zivanovic *et al.*, 2006; Rumajogee *et al.*, 2006).

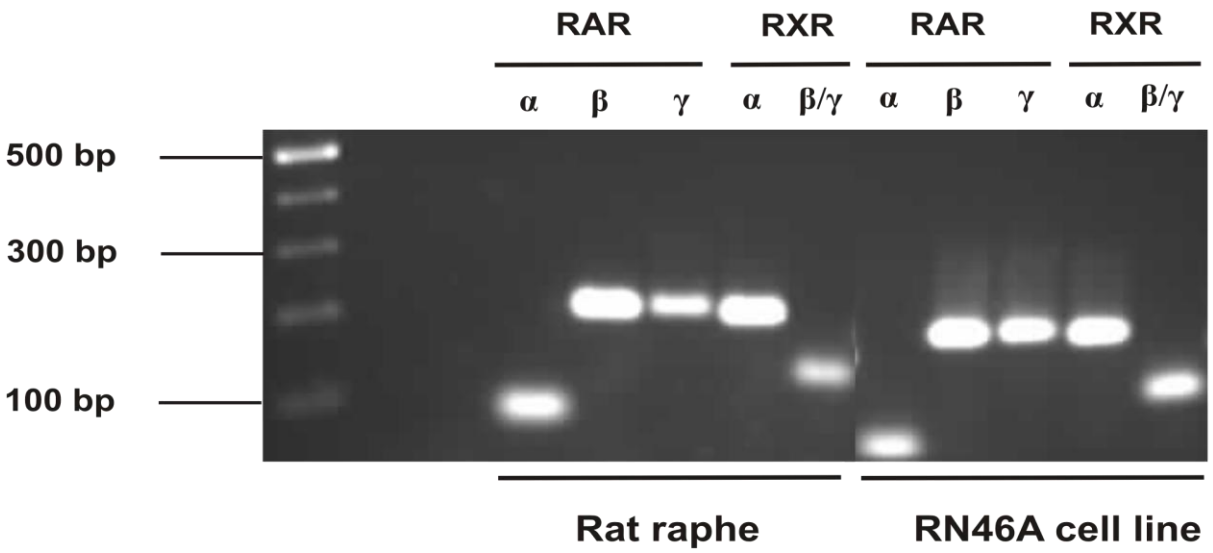

Figure 4.1: Expression of retinoic receptor (RAR) and retinoid 'X' receptor (RXR) mRNAs in rat raphe nuclei tissue and the RN46A-B14 cell line. One-step RT-PCR with gene specific primers (see Table 4.1) allowed the detection of all retinoid receptors investigated both *in vivo* and *in vitro*.

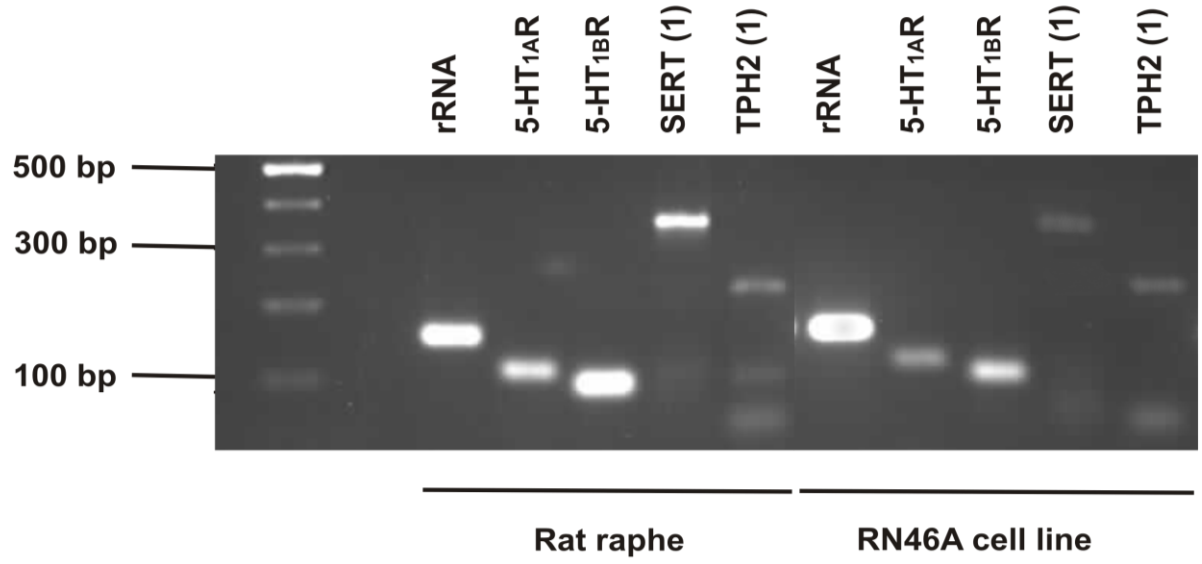

Figure 4.2: Expression of TPH2, SERT, 5-HT_{1A}R and 5-HT_{1B}R mRNAs in rat raphe nuclei tissue and RN46A-B14 cells. One-step RT-PCR with gene specific primers (see Table 4.1) demonstrated the expression of these monoaminergic genes in the RN46A-B14 cell line and the untreated rat raphe nuclei tissue.

The remaining genes of interest, D2DR, MAOA and COMT were similarly analysed by one-step RT-PCR and shown in Figure 4.3. The expression profile of the GOI in the RN46A-B14 cell line was similar to the rat raphe nuclei tissue, with the exception of D2DR expression that was considerably lower *in vitro* compared with *in vivo* expression. Overall, the expression of these monoaminergic components in the retinoid receptor-expressing rat raphe nuclei tissue and RN46A-B14 cells, suggests that D2DR, MAOA and COMT gene expression may be amenable to retinoid regulation.

The expression of all the GOI (with the exception of RARγ, RXRα and RARβ/γ) was also demonstrated in the rat hippocampus via one-step RT-PCR (shown in Figure 4.4). The expression of 5-HT_{1B}R, D2DR and RARβ genes were particularly high, while the expression of SERT, 5-HT_{1A}R, MAOA, COMT and RARα genes was lower. The expression of RARα in the adult mouse hippocampus has been previously reported (Zetterstrom *et al.*, 1999), although interestingly, RARβ expression was not previously detected in this study (Zetterstrom *et al.*, 1999). As expected in the hippocampus, the TPH2 expression appeared to be low as TPH2 is found mainly in the cell bodies of serotonergic neurons in the raphe nuclei (Gutknecht *et al.*, 2009). The findings suggest that the hippocampus may a brain region whereby monoaminergic gene expression is regulated via retinoid signalling components, in a similar manner to that of the raphe nuclei.

Similar studies were performed on the prefrontal cortex and found similar expression of all retinoid receptors and monoaminergic genes (data not shown) and therefore implicate this brain region with monoaminergic gene expression via retinoids. In summary, the results qualitatively confirm the presence of all of the retinoid receptor and monoaminergic GOI investigated in untreated RN46A-B14 cells and raphe nuclei, hippocampal and prefrontal cortex tissue.

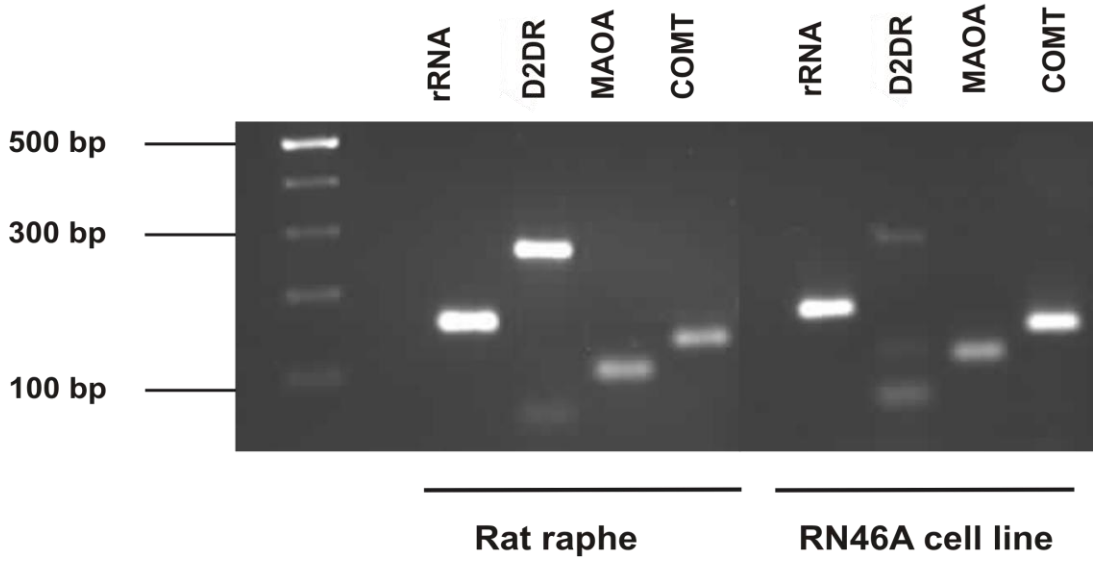

Figure 4.3: Expression of D2DR, MAOA and COMT mRNAs in rat raphe nuclei tissue and RN46A-B14 cells. One-step RT-PCR with gene specific primers (see Table 4.1) demonstrated the presence of these monoaminergic genes in the RN46A-B14 cell line and rat raphe nuclei tissue.

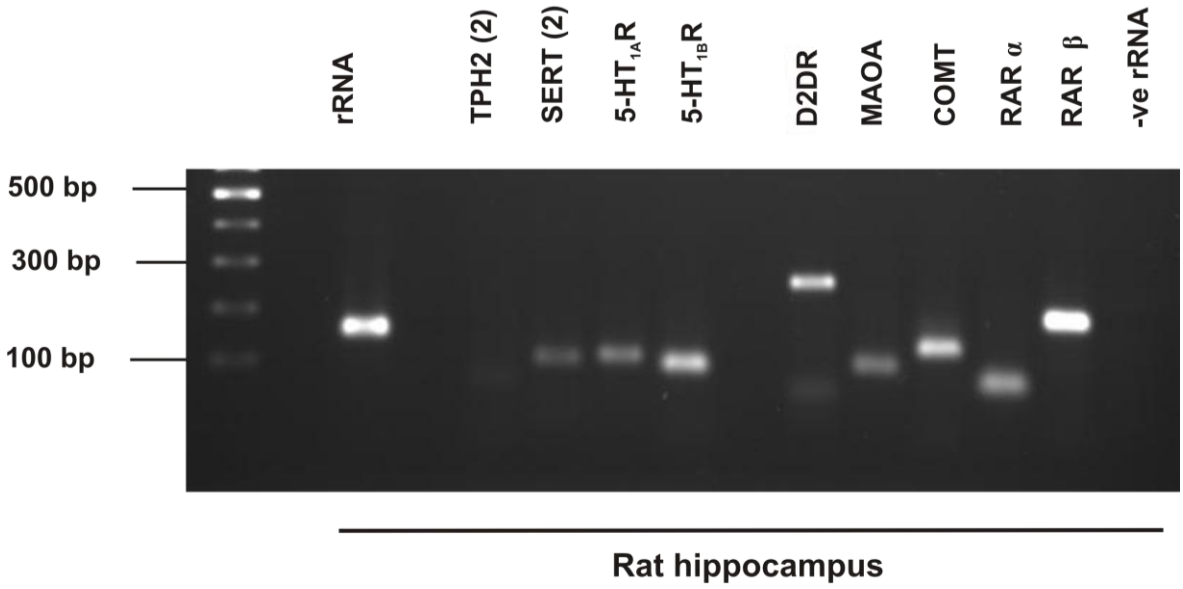

Figure 4.4: Expression of all monoaminergic, RARα and RARβ mRNAs in the adult rat hippocampus. One-step RT-PCR with gene specific primers (see Table 4.1) demonstrated the expression of these monoaminergic and retinoid receptor genes in the rat hippocampal tissue.

4.3.2. The effect *of* 13-*cis*-RA treatment on gene expression

We sought to determine the *in vivo* (1mg/kg/day, 6 weeks, adult and juvenile rats) and *in vitro* effects of 13-*cis*-RA treatment (2.5µM and 10µM, 48 h, RN46A-B14 cells) on gene expression using quantitative real-time RT-PCR and the comparative threshold cycle method. For all quantitative real-time RT-PCR reactions, the same gene specific primers were used as those used for one-step RT-PCR (Table 4.1), with the exception of TPH2 and SERT primers. Previously used TPH2 (1) and SERT (1) and (2) primers did not amplify correctly under quantitative real-time RT-PCR conditions and were replaced with alternative primers TPH2 (2) and SERT (3).

In these quantitative real-time RT-PCR experiments, fluorescent SYBR green was used to detect the PCR products. However, SYBR green preferentially binds to all double stranded DNA. Therefore the melting curve (and melting peak) analyses were conducted in all experiments to determine whether non-specific binding of additional double-stranded DNA products had occurred, evident as smaller secondary peaks on the melting peak analysis. Figures 4.5 and 4.6 show representative amplification curves, melting curve and melting peak analysis which demonstrate the specific amplification of SERT and TPH2 amplicons, respectively, in addition to the amplification of the rRNA amplicon.

Furthermore, the DNA sequence analysis of amplicons using the rRNA, TPH2 (2), SERT (3), 5-HT_{1A}R and D2DR primers revealed that they had a high convergence with their respective gene reference nucleotide sequence. Sequences were analysed using the nucleotide Basic Local Alignment Search Tool program (nBLAST).

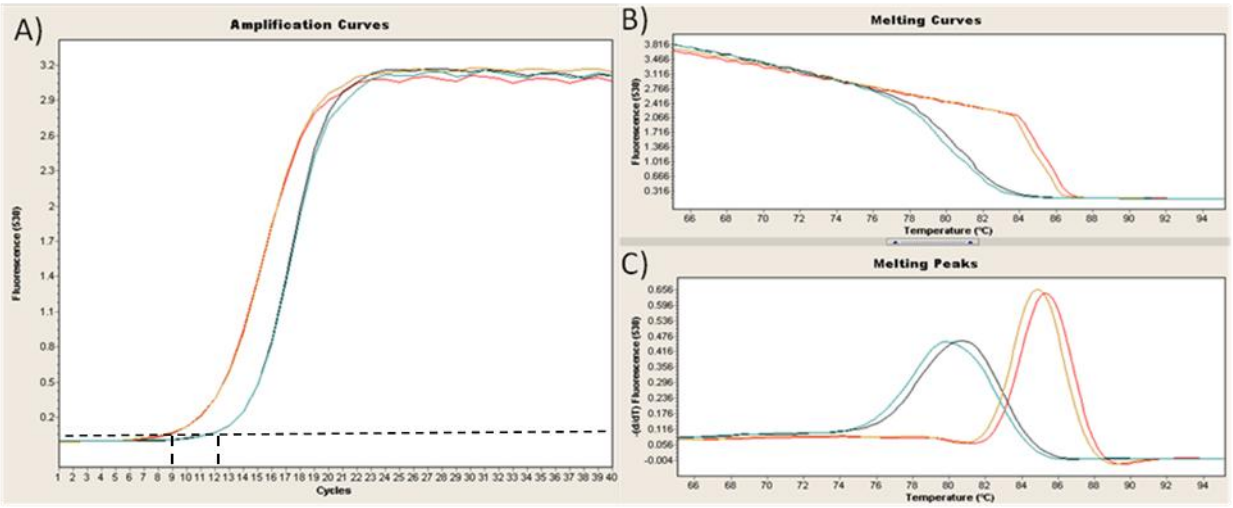

Figure 4.5: Quantitative real-time RT-PCR amplification and melting curves using rRNA and SERT (3) primers. (A) Amplification of housekeeper gene rRNA (two technical repeats, red and orange curves) and SERT (3) (blue and black curves) in vehicle-treated adult rat raphe nuclei tissue. The threshold cycle, or Ct, is the cycle number at which the amplification becomes exponential and is above a baseline signal (see dotted line) and relative changes in gene expression can be calculated. (B) and (C) show melting curves and peaks (rate of change of fluorescence against temperature, df/t) respectively, indicating the differing melting points of rRNA and SERT (3) amplicons and the specificity of the amplification with a single product formed (by a lack of smaller secondary peaks). The Tm for rRNA is 85°C and the Tm for SERT is 80°C.

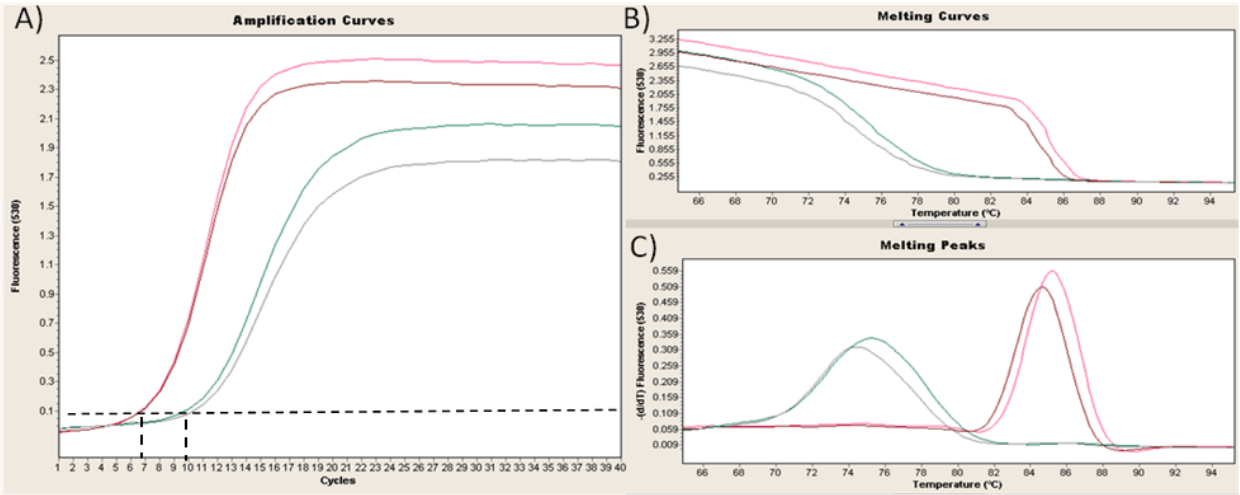

Figure 4.6: Quantitative real-time RT-PCR amplification and melting curves using rRNA and TPH2 (2) primers. (A) Amplification of housekeeper gene rRNA (two technical repeats, pink and brown curves) and TPH2 (2) (green and grey curves) in vehicle-treated juvenile rat raphe nuclei tissue. The threshold cycle, or Ct, is the cycle number at which the amplification becomes exponential and is above a baseline signal (see dotted line) and relative changes in gene expression can be calculated. (B) and (C) show melting curves and peaks (df/t) respectively, indicating the differing melting points of rRNA and TPH2 (2) amplicons and the specificity of the amplification with a single product formed (by a lack of smaller secondary peaks). The Tm for rRNA is 85°C and the Tm for TPH2 is 76°C.

4.3.2.1. The effect of 13-*cis*-RA on gene expression *in vitro*: RN46A-B14 cells

Six biological repeats of RN46A-B14 cells (passage number =7, for all biological repeats) were each plated in 6-well plates (each 6-well plate consisted of two technical repeats for each treatment condition) and gene expression data is from n=3-6 (as some cells were either infected or perished). The gene expression changes in 13-*cis*-RA-treated RN46A-B14 cells (2.5µM and 10µM) were calculated relative to each biological repeat's control (RN46A-B14 cells treated with 0.5% v/v ethanol) and all values were normalized to the rRNA housekeeper gene. The relative fold change was then averaged across all biological repeats for both 2.5µM and 10µM of 13-*cis*-RA treatment. There was no effect of 2.5µM 13-*cis*-RA treatment in TPH2 (4.35 ± 2.10 fold, n=4), SERT (3.30 ± 1.15 fold, n=4), 5-HT_{1A}R (1.91 ± 0.74 fold, n=5) and 5-HT_{1B}R (2.34 ± 0.63 fold, n=3) gene expression (P>0.05 in all cases, unpaired t-test and shown in Figure 4.7), although there was a trend for increased 5-HT_{1B}R expression after 2.5µM treatment (P=0.068). Similarly, there was no effect of higher concentrations of 13-*cis*-RA (10µM) in TPH2 (1.88 ± 0.51 fold, n=4), SERT (2.14 ± 0.66 fold, n=4), 5-HT_{1A}R (2.34 ± 1.13, n=5) and 5-HT_{1B}R (1.57 ± 0.61fold, n=3) gene expression (P>0.05 in all cases, unpaired t-test and shown in Figure 4.7) compared with vehicles and no effect between the 2.5µM and 10µM concentrations of 13-*cis*-RA used. The results were similar to an earlier study by our group that had shown that mRNA levels of SERT and 5-HT_{1A}R in the RN46A-B14 cell line were unaltered by 48 h of 2.5µM and 10µM 13-*cis*-RA treatment (O'Reilly *et al.*, 2007).

There was a significant effect of 10µM 13-*cis*-RA treatment on D2DR gene expression (n=5, P=0.047, unpaired t-test) and a trend for D2DR increases at the lower concentration (n=6, P=0.084, unpaired t-test, Figure 4.8). Previous studies have shown the presence of a RARE in the promoter region of the D2DR gene (Samad *et al.*, 1997), so it is perhaps unsurprising that retinoid treatment in the form of 13-*cis*-RA induces D2DR gene upregulation (therefore acting as a positive control). Meanwhile, gene expression changes for MAOA (n=4) and COMT (n=4) in RN46A-B14 cells treated with 2.5µM of 13-*cis*-RA were 1.07 ± 0.05 and 1.01 ± 0.12 fold, respectively, whereas RN46A-B14 cells treated with 10µM of 13-*cis*-RA had MAOA (n=4) and COMT (n=4) gene expression changes

of 3.22 ± 0.95, 1.83 ± 0.55 and 4.21 ± 3.13 fold, respectively (Figure 4.8). Both concentrations of 13-*cis*-RA treatment (2.5µM and 10µM) had no significant effect on COMT or MAOA gene expression (P>0.05 in all cases, unpaired t-test).

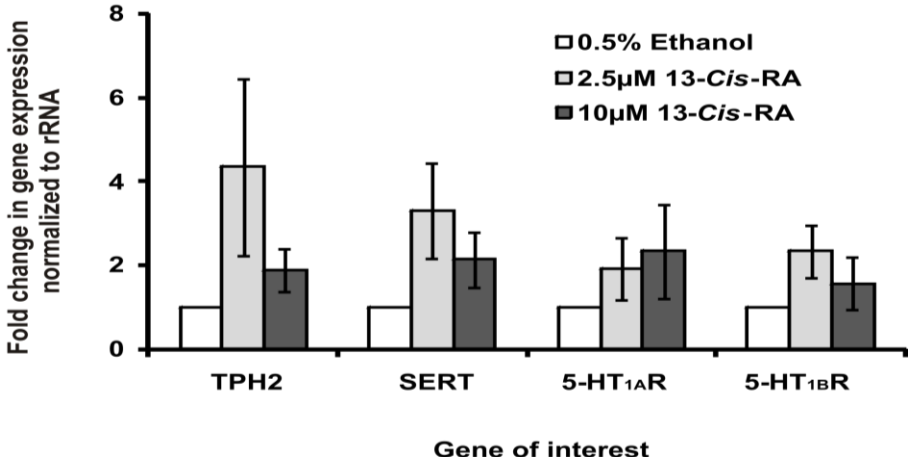

Figure 4.7: Relative fold changes in TPH2, SERT, 5-HT_{1A}R and 5-HT_{1B}R gene expression in RN46A-B14 cells treated with 13-*cis*-RA. RN46A-B14 cells were treated with either vehicle (0.5% ethanol, n=3-5, open bars), 2.5µM 13-*cis*-RA (n=3-5, light grey bars) or 10µM 13-*cis*-RA (n=3-5, dark grey bars). Gene changes are relative to 0.5% ethanol treated cells (=1) and normalized to housekeeper gene rRNA. Error bars indicate SEM.

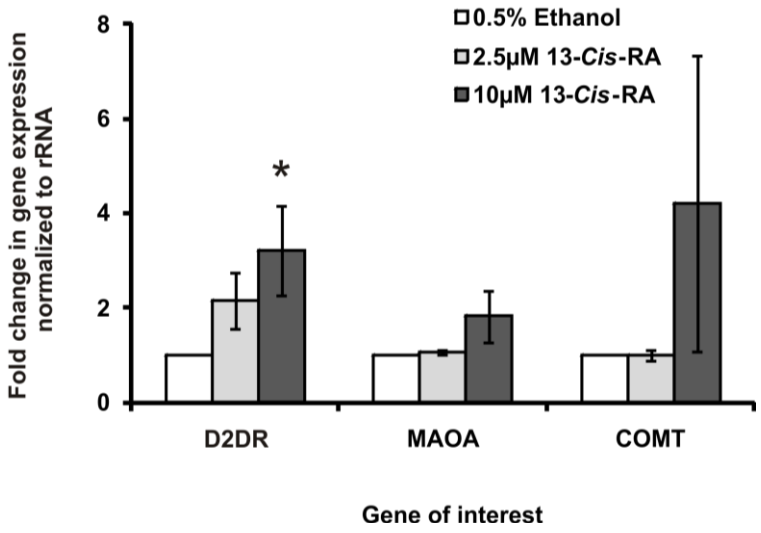

Figure 4.8: Relative fold changes in D2DR, MAOA and COMT gene expression in RN46A-B14 cells treated with 13-*cis*-RA. RN46A-B14 cells were treated with either vehicle (0.5% ethanol, n=4-6, open bars), 2.5µM 13-*cis*-RA (n=4-6, light grey bars) or 10µM 13-*cis*-RA (n=4-6, dark grey bars). Gene changes are relative to 0.5% ethanol treated cells (=1 fold change) and normalized to housekeeper gene rRNA. * denotes P=0.035, unpaired t-test. Error bars indicate SEM.

Retinoid treatment should induce greater gene transcription of its own receptors since both RARα and RARβ contain RAREs (Brand *et al.*, 1988; Petkovich *et al.*, 1987). Surprisingly, in these experiments 13-*cis*-RA did not induce an increase in the expression of RARα and RARβ (Figure 4.9). Gene expression changes for RARα (n=4) and RARβ (n=3) in RN46A-B14 cells treated with 2.5μM of 13-*cis*-RA were 1.10 ± 0.25 and 0.49 ± 0.08 fold, respectively (shown in Figure 4.9), while gene expression changes in RN46A-B14 cells treated with 10μM of 13-*cis*-RA for RARα (n=4) and RARβ (n=3) were 2.03 ± 0.65 and 0.65 ± 0.13 fold, respectively. In all cases, both concentrations of 13-*cis*-RA had no significant effect on gene expression (P>0.05, unpaired t-test) except for significantly reducing RARβ expression at 2.5μM of 13-*cis*-RA (P=0.031, unpaired t-test).

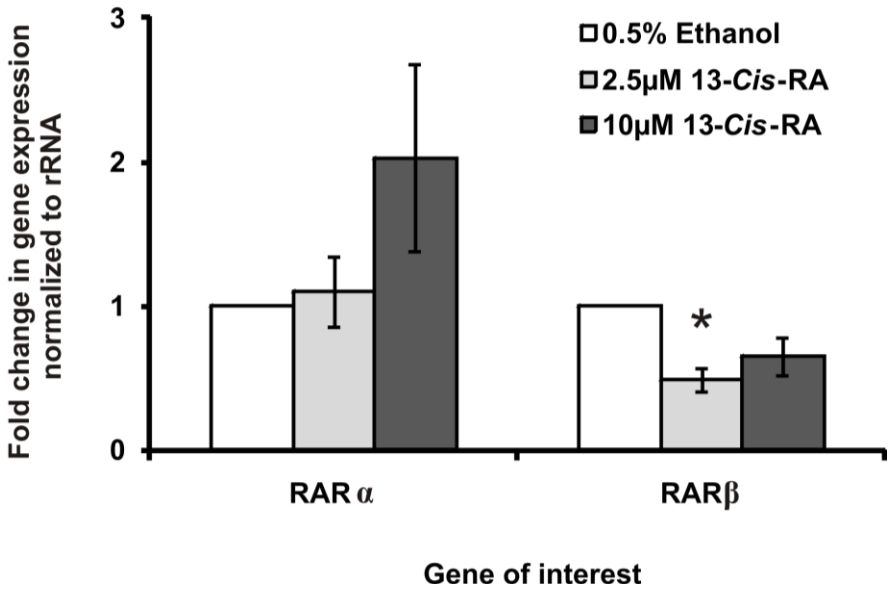

Figure 4.9: Relative fold changes in RARα and RARβ gene expression in RN46A-B14 cells treated with 13-*cis*-RA. RN46A-B14 cells were treated with either vehicle (0.5% ethanol, n=3/4, open bars), 2.5μM 13-*cis*-RA (n=3/4, light grey bars) or 10μM 13-*cis*-RA (n=3/4, dark grey bars). Gene changes are relative to 0.5% ethanol treated cells (=1 fold change) and normalized to housekeeper gene rRNA. * denotes P=0.031, unpaired t-test. Error bars indicate SEM.

4.3.2.2. The effect of 13-*cis*-RA on gene expression *in vivo*: adult and juvenile rat raphe nuclei

The gene expression changes mediated by 6 weeks of treatment with 13-*cis*-RA (1mg/kg/day, dissolved in vehicle) were measured in the raphe nuclei of adult and juvenile rats using quantitative

real-time RT-PCR and the comparative threshold cycle method. To quantify relative changes in gene expression, the average cycle threshold number of the GOI in raphe nuclei of all adult rats treated with 13-*cis*-RA (n=4) were first normalised to the average cycle threshold number of the housekeeper gene rRNA of the same rats and then compared to the averaged cycle threshold number of the GOI in the raphe nuclei of all adult rats treated with vehicle (1ml/kg/day, 1:1 saline:DMSO, n=4), normalised to the housekeeper gene rRNA. Averages were used as a single 13-*cis*-RA-treated rat cannot be directly paired with a single vehicle-treated rat for comparison, as each animal represents a disparate biological entity, unlike the experiment with RN46A-B14 cells, whereby each biological repeat has its own control for comparison. An identical arrangement was employed for juvenile rat raphe nuclei analysis. The primers used for quantitative real-time RT-PCR were the same as those used previously for *in vitro* quantitative real-time RT-PCR analysis, although RARβ and COMT were omitted because RARβ had failed to act as a positive control for retinoid treatment and COMT is poorly expressed in the raphe nuclei.

The treatment of adult and juvenile rats with 13-*cis*-RA for 6 weeks had no significant effect on the relative upregulation/downregulation of the GOI in the raphe nuclei ($P>0.05$, unpaired t-test, Figure 4.10). The fold change of TPH2 (n=4), SERT (n=4), 5-HT_{1A}R (n=4), 5-HT_{1B}R (n=5), D2DR (n=4), MAOA (n=4) and RARα (n=4) gene expression in the raphe of adult rats treated with 13-*cis*-RA was 5.62 ± 12.37, 3.79 ± 5.68, 0.14 ± 2.12, 8.69 ± 16.83, 2.48 ± 12.68, 0.72 ± 1.86 and 4.11 ± 41.21, respectively, relative to the fold change of vehicle-treated rats (all set to 1 fold ± 1.54, 8.16, 15.88, 5.33, 12.67, 12.91 and 19.34, respectively, Figure 4.10A). The standard deviations for the averaged C_T numbers of the GOI and rRNA in both vehicle and 13-*cis*-treated adult rats were high (particularly as the square root of the sum of the standard deviations squared are calculated and then exponentiated to the base 2), leading to large standard errors seen in Figure 4.10A. In contrast, more consistent data was obtained from the juvenile rats (Figure 4.10 B). The relative fold change of TPH2 (n=4), SERT (n=4), 5-HT_{1A}R (n=4), 5-HT_{1B}R (n=4), D2DR (n=4), MAOA (n=4) and RARα (n=4) gene expression in the raphe nuclei of juvenile rats treated with 13-*cis*-RA was 1.70 ± 1.14, 1.50 ± 0.78, 0.86 ± 0.46, 1.03 ± 0.39, 1.24 ± 0.31, 1.26 ± 0.29 and 1.15 ± 0.19, respectively (relative to vehicle-treated rats

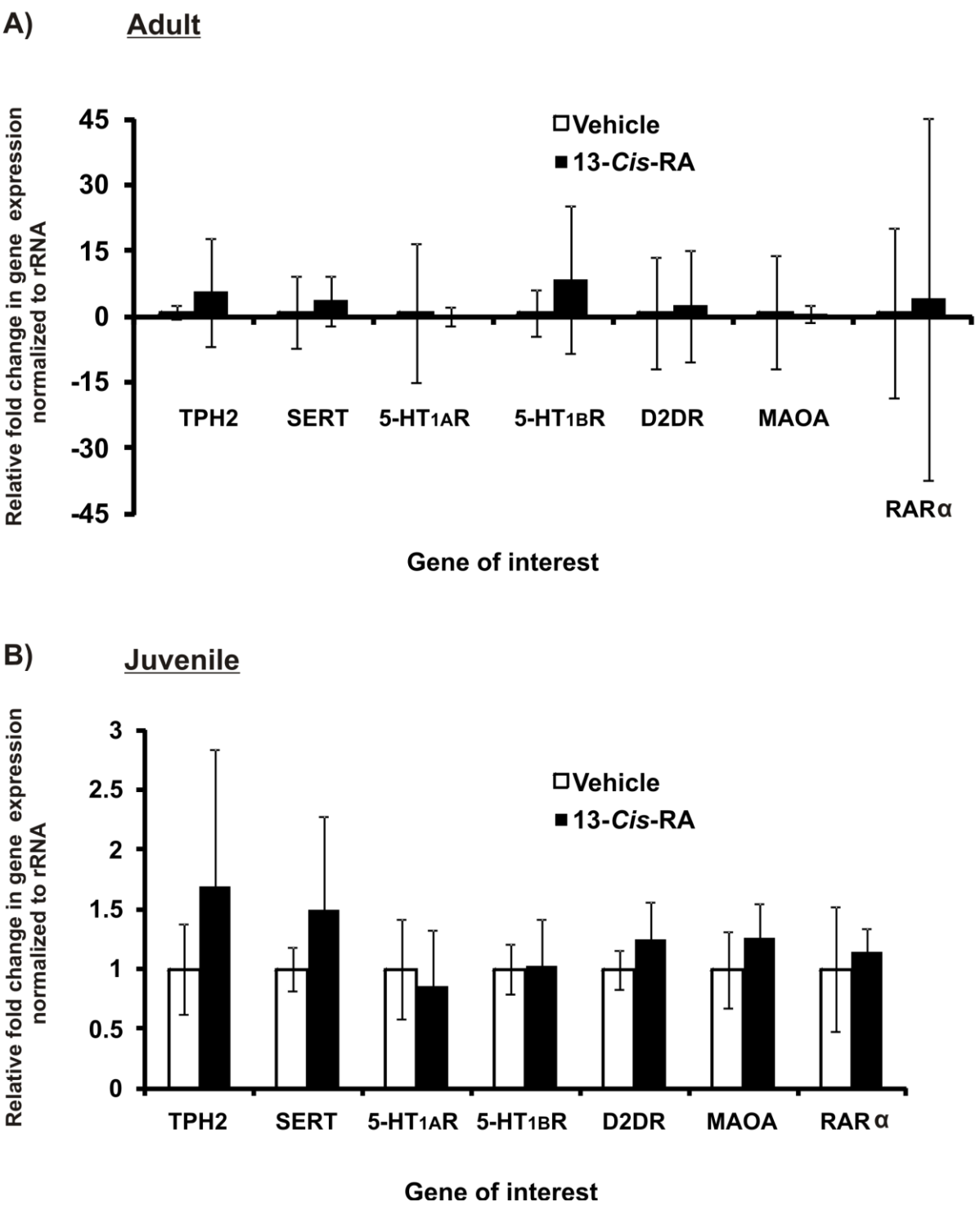

Figure 4.10: The effect of 13-*cis*-RA treatment on gene expression in the raphe nuclei of adult and juvenile rats. Real-time RT-PCR was performed on the raphe nuclei tissue of adult (A) and juvenile (B) rats treated for 6 weeks with 13-*cis*-RA. To calculate relative changes, the comparative threshold cycle method compared 13-*cis*-RA-treated rats (n=4, closed bars) with vehicle-treated rats (n=4, open bars) and normalized to the housekeeper gene rRNA. Error bars are ± SEM.

which were set as a 1 fold change ± 0.38, 0.19, 0.42, 0.21, 0.16, 0.32 and 0.52, respectively). 13-*Cis*-RA treatment did not affect GOI gene expression in juvenile rats (P>0.05, unpaired t-test) and as in the *in vitro* study, there was no significant upregulation of RARα in both adult and juvenile rat raphe nuclei. This result is surprising since retinoid treatment causes activation of retinoid receptors that in turn, are able to increase retinoid receptor gene expression (Brand *et al.*, 1988; Petkovich *et al.*, 1987). The inability of 13-*cis*-RA to affect D2DR gene expression in the adult and juvenile rat raphe nuclei *in vivo* was also surprising for the same reason.

4.3.2.3. The effect of 13-*cis*-RA on gene expression *in vivo*: adult and juvenile rat hippocampus

Gene expression changes were also examined in the hippocampus of adult and juvenile rats to determine the effects of chronic 13-*cis*-RA treatment. The methods and analysis were identical to those used previously for quantitative real-time RT-PCR analysis of rat raphe nuclei tissue. The treatment of adult and juvenile rats with 13-*cis*-RA had no significant effect on the relative upregulation/downregulation of the GOI in the hippocampus (P>0.05, unpaired t-test, Figure 4.11). The fold change of TPH2 (n=4), SERT (n=4), 5-HT_{1A}R (n=4), 5-HT_{1B}R (n=4), D2DR (n=4), MAOA (n=4) and RARα (n=4) genes in the hippocampus of adult rats treated with 13-*cis*-RA was 1.11 ± 0.18, 1.31 ± 1.04, 0.86 ± 0.16, 0.74 ± 0.13, 1.18 ± 0.22, 1.06 ± 0.99 and 1.36 ± 0.42, respectively (relative to vehicle-treated rats set as a 1 fold change ± 0.13, 0.94, 0.13, 0.28, 0.50, 0.91 and 0.32, respectively, Figure 4.11A). Meanwhile, the fold change of TPH2 (n=4), SERT (n=4), 5-HT_{1A}R (n=4), 5-HT_{1B}R (n=4), D2DR (n=4), MAOA (n=4) and RARα (n=4) genes in the hippocampus of juvenile rats treated with 13-*cis*-RA was 1.84 ± 0.46, 1.18 ± 0.31, 1.07 ± 0.20, 1.04 ± 0.24, 1.67 ± 0.86, 1.16 ± 1.40 and 1.30 ± 0.79 (relative to vehicle-treated rats set as a 1 fold change ± 0.42, 0.13, 0.12, 0.13, 0.63, 0.94 and 0.40, respectively, Figure 4.11B). There is a potentially mild trend for increased TPH2 gene expression in the hippocampus of 13-*cis*-RA-treated juvenile rats (P=0.13, unpaired t-test).

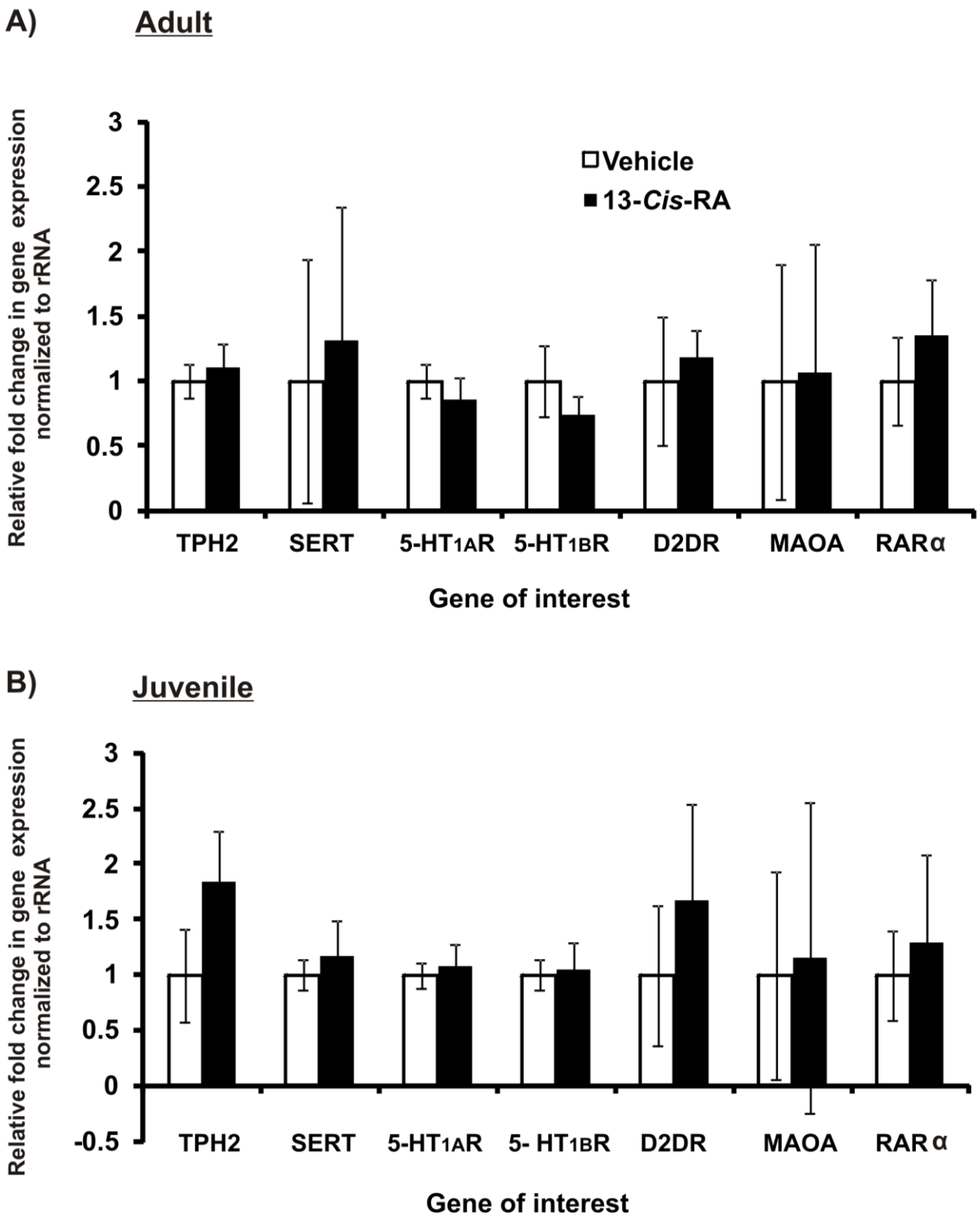

Figure 4.11: The effect of 13-*cis*-RA treatment on gene expression in the hippocampus of adult and juvenile rats. Real-time RT-PCR was performed on hippocampal tissue of adult (A) and juvenile (B) rats. To calculate relative changes, the comparative threshold cycle method compared 13-*cis*-RA-treated rats (n=4, closed bars) with vehicle-treated rats (n=4, open bars) and normalized to the housekeeper gene rRNA. Error bars are ± SEM.

4.3.3. The *in vitro* and *in vivo* effects *of* 13-*cis*-RA treatment on protein levels in the rat raphe nuclei

Following the gene expression studies, we next sought to compare whether the protein levels of certain monoaminergic components were altered by 13-*cis*-RA treatment. Semi-quantitative Western blotting was used to assess the protein levels of TPH2, SERT, 5-HT_{1A}R and D2DR (positive control) in the RN46A-B14 rat raphe cell line and microdissected rat raphe nuclei tissue. These proteins were investigated based on a) gene expression data that had shown D2DR gene expression increased significantly *in vitro* and is also a positive control of 13-*cis*-RA treatment, b) a previous study by our group that had shown that protein levels of SERT and 5-HT_{1A}R in the RN46A-B14 cell line were elevated after 48 h of 2.5µM and 10µM 13-*cis*-RA treatment (O'Reilly *et al.*, 2007) and c) the putative possibility that TPH2 gene expression may have been raised *in vitro* and in 13-*cis*-RA-treated juvenile rats (see Figure 4.7, 4.10 and 4.11) and may therefore be upregulated at the protein level.

Validation of immunolabelling conditions and specificity were conducted for anti-TPH2, anti-SERT, anti-5-HT_{1A}R and anti-D2DR antibodies in untreated rat brain tissue (prefrontal cortex, hippocampus, striatum and raphe nuclei) in parallel with a non-neuronal MDA-MB-468 cell line acting as a negative control (Figure 4.12). The anti-TPH2 antibody detected a single protein band at 56kDa, which corresponds with the known molecular weight of TPH2. As would be expected, TPH2 appears to be highly enriched in the raphe nuclei tissue compared with the prefrontal cortex and hippocampus and no bands were detected in the non-neuronal cell line MDA-MB-468. Bands corresponding to TPH2, SERT, 5-HT_{1A}R, D2DR and β-actin protein were clearly observed in all tissues tested with the exception of D2DR protein in the rat raphe nuclei tissue. This was is in concordance with a previous *in situ* hybridization study that had shown low levels of D2DR mRNA transcript in the rat dorsal raphe (Martin-Ruiz *et al.*, 2001). All validation blots were conducted with a total of 20µg of protein and therefore all subsequent experiments similarly used 20µg (for raphe and hippocampal samples) or 7µg (for the RN46A-B14 cell line).

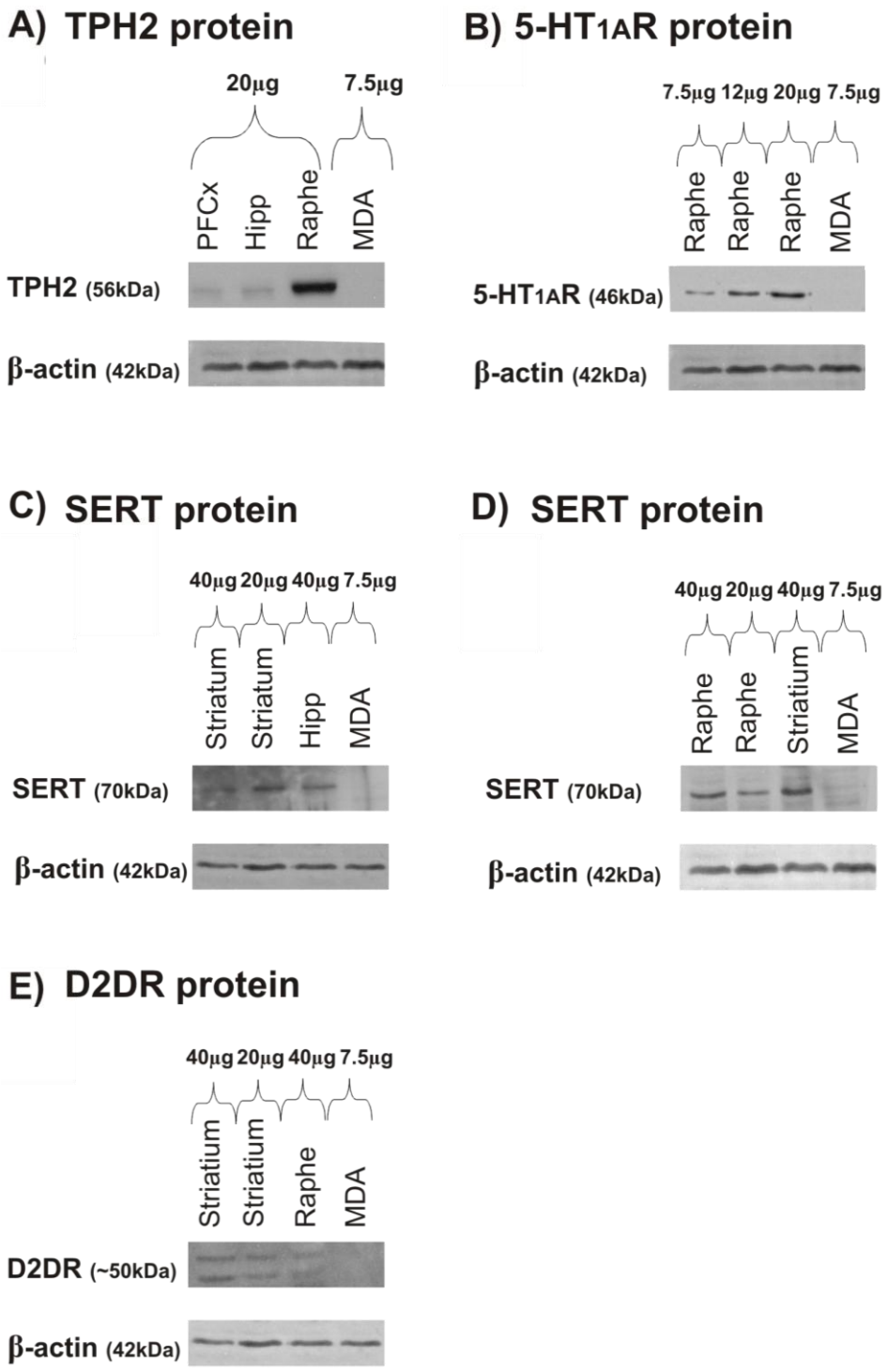

Figure 4.12: Validation of primary antibodies used for semi-quantitative Western blot analysis. A) rabbit polyclonal anti-TPH2 antibody was used to detect TPH2 (specific bands, 56kDa) in prefrontal cortex (PFCx), hippocampus (Hipp) and raphe nuclei tissue. Immunolabelling was not seen in the non-neuronal human breast adenocarcinoma MDA-MB-468 cell line (MDA, negative control). B) immunolabelling with rabbit polyclonal anti-5-HT_{1A}R antibody in increasing concentrations of raphe nuclei tissue (bands at 46kDa). C) and D) rabbit polyclonal anti-SERT antibody immunolabelling of rat raphe nuclei, striatum, hippocampus (70kDa bands). E) immunolabelling with rabbit polyclonal anti-D2DR in rat striatum and raphe nuclei (bands at 51kDa and 48kDa). All blots were stripped and re-probed with anti-β-actin antibody as a loading control (bands at 42kDa).

4.3.3.1. The effect of 13-*cis*-RA on protein levels *in vitro*: RN46A-B14 cells

Gene expression data from RN46A-B14 cells treated with 13-*cis*-RA revealed no overall change in gene expression of the GOI with the exception of D2DR, which was significantly elevated at 2.5µM (Figure 4.8). Meanwhile, an earlier study conducted by our group was able to show that protein levels of SERT and 5-HT_{1A}R in the RN46A-B14 cell line were found to be elevated after 48 h of 2.5µM and 10µM 13-*cis*-RA treatment (O'Reilly *et al.*, 2007). Furthermore, TPH2 protein levels may be altered given that TPH2 gene expression data suggests an increase in 13-*cis*-RA-treated juvenile rat raphe and hippocampus, as well as *in vitro*. We have therefore conducted semi-quantitative Western blotting to analyse the potential effects of 13-*cis*-RA treatment (control, 2.5µM and 10µM) in the RN46A-B14 cell line on SERT, 5-HT_{1A}R, D2DR and TPH2 protein levels. In all cases, a technical repeat was introduced by running an additional Western blot in parallel.

The analysis of the Western blots failed to reveal bands for the D2DR protein that was presumably due to the low levels of D2DR in the raphe cell line (data not shown). Unexpectedly, bands for the TPH2, 5-HT_{1A}R, SERT and β-actin protein were poorly visualized. The low level of β-actin appears to suggest that the total quantity of protein was insufficient for reliable detection of bands via immunolabelling, despite using 17µg of protein/lane (protein samples were concentrated using Amicon 4 ultra centrifuge columns, Millipore). It was therefore not possible to perform densitometric analysis using Lab Image and in turn, meant we were unable to measure the *in vitro* effects of 13-*cis*-RA at the protein level.

4.3.3.2. The effect of 13-*cis*-RA on protein levels *in vivo*: adult and juvenile rat raphe nuclei

Although gene expression data did not reveal any significant effects of 13-*cis*-RA treatment in gene expression in both the adult and juvenile rat raphe nuclei, so we sought to quantify protein levels of TPH2, SERT, 5-HT_{1A}R and D2DR. In all cases, a technical repeat was introduced by running an additional Western blot in parallel. 13-*Cis*-RA treatment did not significantly alter protein levels in

adult rat raphe nuclei tissue (P>0.05, unpaired t-test, Figure 4.13). Figure 4.13A and C shows TPH2, SERT and 5-HT_{1A}R antibody-specific bands in adult and juvenile rat raphe nuclei tissue. The protein expression levels of TPH2 (n=4), SERT (n=4) and 5-HT_{1A}R (n=3) in 13-*cis*-RA-treated adult rat raphe nuclei was calculated to be 80.5 ± 18.2%, 79.7 ± 18.7% and 85.0 ± 7.4%, respectively. All values are a percentage of loading control and normalized to vehicle protein expression levels (set as 100 ± 31.4%, 30.9% and 20.5%, respectively, n=4 for TPH2 and SERT, and n=3 for 5-HT_{1A}R).

However, there was a trend for reduced TPH2 protein levels in the juvenile raphe nuclei of 13-*cis*-RA-treated rats (P=0.058, unpaired t-test, Figure 4.13D), while no significant changes were found in SERT and 5-HT_{1A}R protein levels. The protein expression levels of TPH2 (n=4), SERT (n=4) and 5-HT_{1A}R (n=4) in 13-*cis*-RA-treated juvenile rat raphe nuclei was calculated to be 75.0 ± 16.1%, 95.5 ± 5.2% and 95.5 ± 5.2%, respectively. All values are a percentage of loading control and normalized to vehicle protein expression levels (set as 100 ± 75.0%, 95.5% and 95.55%, respectively, n=4 in all cases). The protein level of D2DR within the raphe nuclei of adult and juvenile rats was too low to accurately perform densitometric analysis.

4.3.4. The effect of 13-*cis*-RA on protein levels *in vivo*: adult and juvenile rat hippocampus

We sought to quantify the same proteins of interest as those in Chapter 4.3.3.2 in the hippocampus of vehicle and 13-*cis*-RA-treated adult (TPH2 and D2DR only) and juvenile rats (TPH2, SERT, 5-HT_{1A}R and D2DR) given it may be this region, as opposed to the raphe nuclei, which may be sensitive to the effects of 13-*cis*-RA. Furthermore, the gene expression data suggested a mild trend for increased expression of TPH2 within the hippocampus (see Figure 4.11B) that may become evident at the protein level. 13-*Cis*-RA treatment had no effect on TPH2 or D2DR protein levels in the adult rat hippocampus (P>0.05, unpaired t-test, Figure 4.14B). The protein levels of TPH2 (n=4) and D2DR (n=4) in 13-*cis*-RA-treated adult rat hippocampus were calculated to be 132.1 ± 10.9% and 98.7 ± 27.6%, respectively.

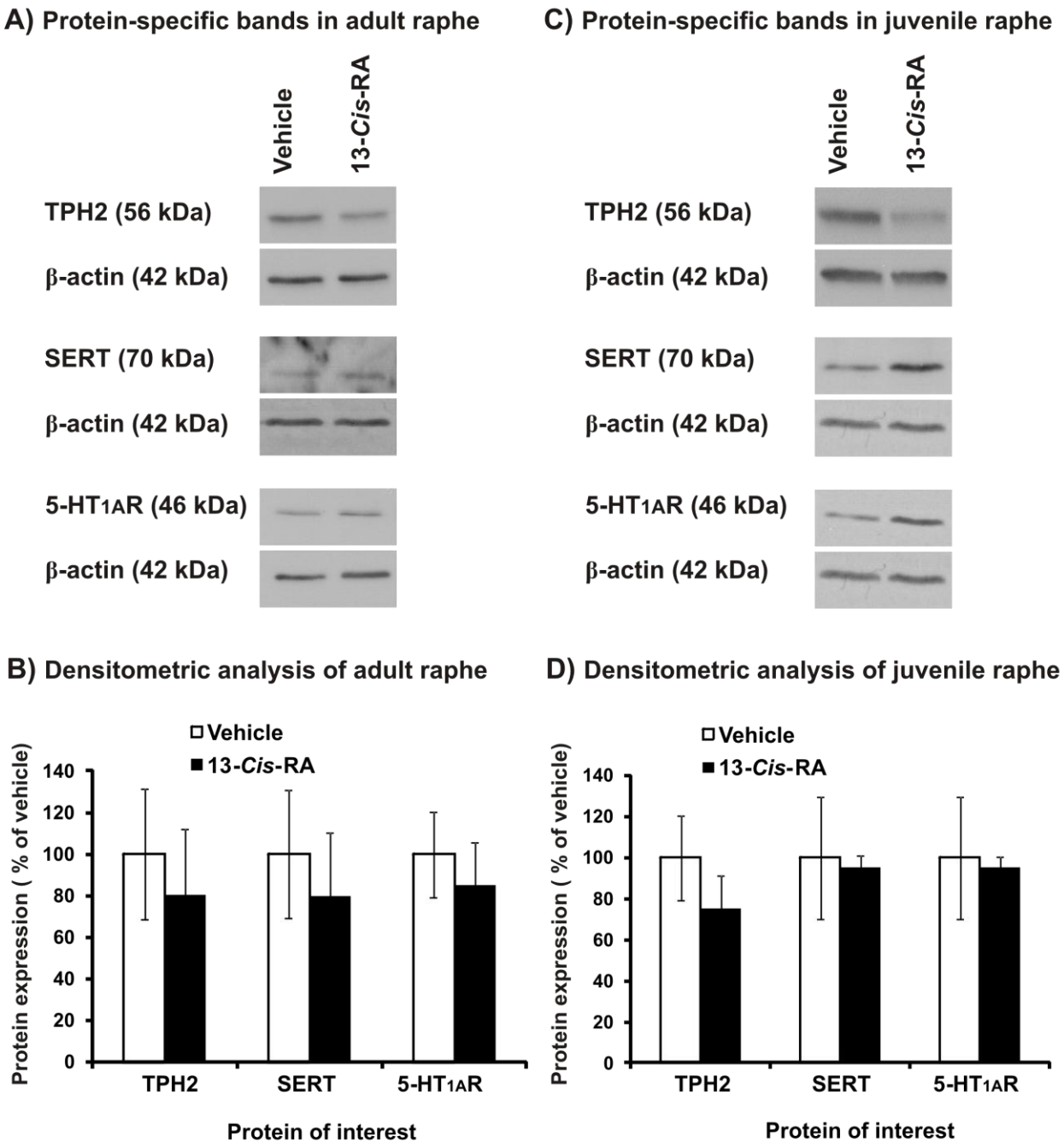

Figure 4.13: The effect of 13-*cis*-RA treatment on TPH2, SERT and 5-HT_{1A}R protein levels in adult and juvenile rat raphe nuclei tissue. A) and C) show the protein-specific bands of TPH2, SERT and 5-HT_{1A}R (with β-actin as a loading control) in the raphe nuclei of adult and juvenile rats, respectively, treated with either vehicle or 13-*cis*-RA. B) Densitometric analysis of Western blot bands in adult rat raphe nuclei. Values of protein changes in 13-*cis*-RA-treated rats (n=3-4, closed bars) are relative to β-actin and normalized to vehicle-treated rat values (n=3-4, open bars). D) Densitometric analysis of Western blot bands in 13-*cis*-RA-treated juvenile rat raphe nuclei (n=4, closed bars) relative to vehicle-treated juvenile rat raphe nuclei (n=4, open bars). Representative immunoblots are shown. Error bars indicate standard deviation.

All values are a percentage of loading control and normalized to vehicle-treated protein levels (set as 100 ± 10.9% and 27.6%, respectively, n=4 in both cases)

In juvenile rats, there were no significant changes in TPH2, SERT and 5-HT_{1A}R protein levels in the hippocampus after 13-*cis*-RA treatment (Figure 4.14D). The protein levels of TPH2 (n=3), SERT (n=3), 5-HT_{1A}R (n=3) and D2DR (n=3) in 13-*cis*- RA-treated juvenile rat hippocampus were calculated to be 111.5 ± 10.5%, 105.2 ± 22.1%, 99.7 ± 8.5% and 134.7 ± 11.2%, respectively. All values are a percentage of loading control and normalized to vehicle-treated protein (set as 100 ± 33.4%, 7.6%, 11.1% and 11.4%, respectively, n=4 in all cases).). 13-*Cis*-RA treatment was found to significantly increase D2DR protein levels in the juvenile hippocampus (*P*=0.017, unpaired t-test). The rise in D2DR gene and protein expression following retinoid treatment suggests increases in both D2DR gene transcription and the subsequent translation to D2DR protein in the hippocampus of juvenile rats.

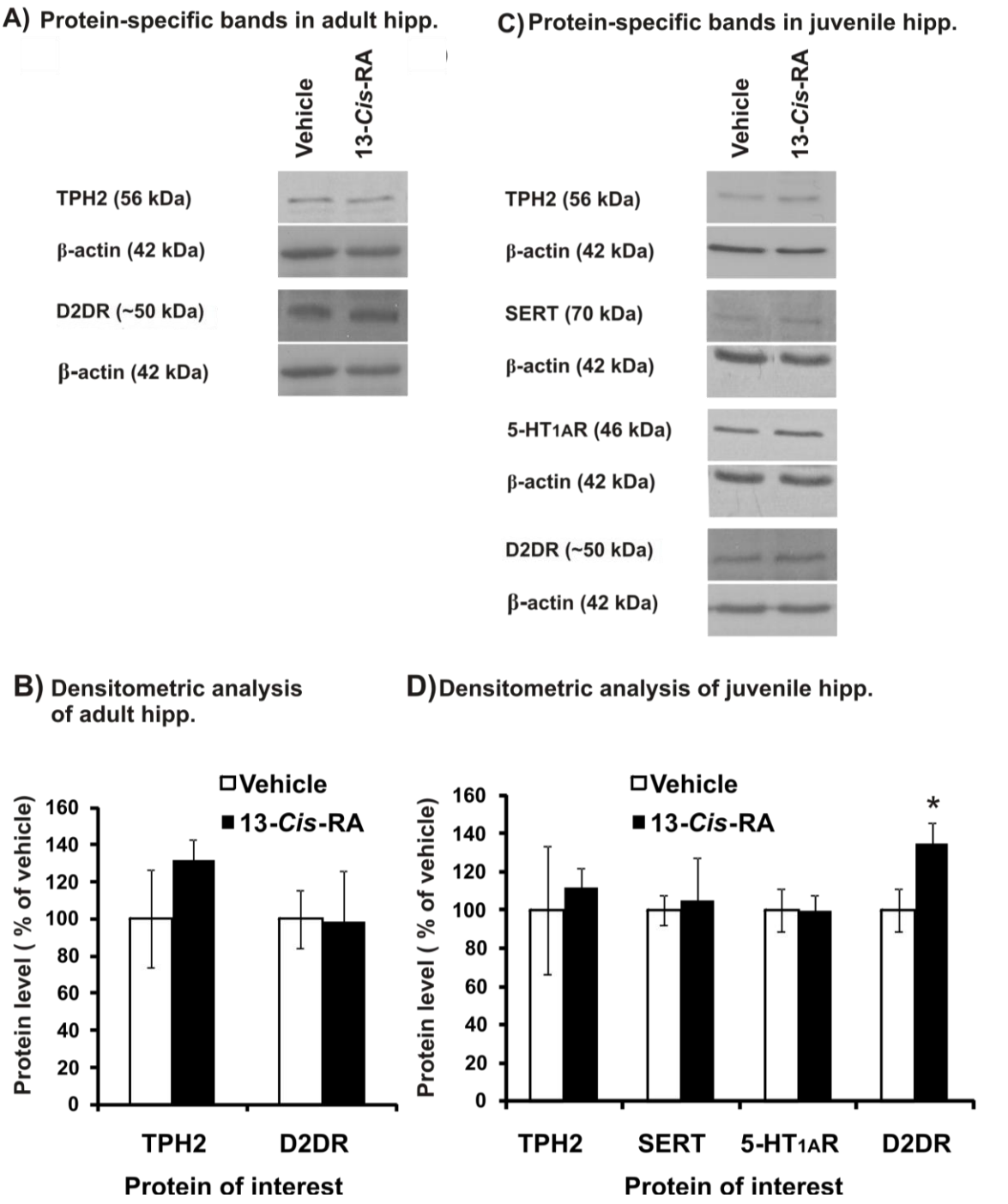

Figure 4.14: The effect of 13-*cis*-RA treatment on TPH2, SERT, 5-HT_{1A}R and D2DR protein levels in adult and juvenile rat hippocampus. A) shows the protein-specific bands of TPH2 and D2DR (with β-actin loading control) in hippocampus of vehicle and 13-*cis*-RA-treated adult rats. B) Densitometric analysis of Western blot bands in adult rat hippocampus, with changes in 13-*cis*-RA-treated rats (n=4, closed bars) given relative to β-actin and normalized to vehicle-treated rat values (n=4, open bars). C) shows the protein-specific bands of TPH2, SERT, 5-HT_{1A}R and D2DR (with β-actin loading control) in vehicle and 13-*cis*-RA-treated juvenile rats. D) Densitometric analysis of blot bands in juvenile rat hippocampus with changes in 13-*cis*-RA-treated rats (n=3, closed bars) given relative β-actin and normalized to vehicle-treated rat values (n=4, open bars). Representative immunoblots are shown. Hipp = hippocampus. Error bars indicate standard deviation.

4.4. Discussion

The work in this chapter sought to further our understanding of effects of 13-*cis*-RA treatment on monoaminergic gene expression and protein levels. The results are summarised in Table 4.3. The major findings were *i)* that RARα, RARβ, RARγ, RXRα and RXRβ/γ genes were expressed in the rat raphe nuclei tissue, along with an analogous *in vitro* cell line, RN46A-B14, and in the rat hippocampus, *ii)* D2DR gene expression was significantly elevated in the RN46A-B14 cell line and D2DR protein levels were significantly elevated in the juvenile rat hippocampus following 13-*cis*-RA treatment, *iii)* the lack of any effect of 13-*cis*-RA on SERT and 5-HT_{1A}R protein levels, in contrast to previously reported findings, *iv)* trends for an increase in TPH2 gene expression in the juvenile rat hippocampus and a reduction in TPH2 protein levels in the juvenile raphe nuclei following 13-*cis*-RA treatment and *v)* the lack of any effect of 13-*cis*-RA on MAOA, 5-HT_{1B}R and RARα gene expression in the rat raphe nuclei, rat hippocampus and RN46A-B14 cell line, although there was a trend for increased 5-HT_{1B}R gene expression in the RN46A-B14 cell line.

4.4.1. RAR and RXR expression in the rat raphe nuclei, hippocampus and RN46-A-B14 cell line

All of our genes of interest, including all retinoid receptors subtypes, were expressed in untreated rat raphe/hippocampus and the RN46A-B14 cell line as determined by one-step RT-PCR. The presence of RARs and RXRs in the rat raphe nuclei is a novel finding, as it has only been previously reported that CRABP I mRNA expression is expressed in the raphe nuclei of adult mice (Zetterstrom *et al.*, 1999) and suggests this brain region is an active site of retinoid signalling. Furthermore, similar to the rat raphe nuclei, all RAR and RXR genes were expressed in the RN46A-B14 cell line, thereby confirming its suitability as an *in vitro* model in the study of retinoid-signalling in the raphe nuclei. In the adult rat hippocampus, both RARα and RARβ gene expression was observed, whereas it had been previously reported that RARα, but not RARβ, was expressed in the adult mouse hippocampus (Zetterstrom *et al.*, 1999). Overall, the presence of retinoid-signalling components, along with the

			TPH2	SERT	5-HT$_{1A}$R	D2DR	5-HT$_{1B}$R	COMT	MAO-A	RARα	RARβ
Gene expression	RN46-B14 cell line		↔	↔	↔	↑*	↑	↔	↔	↔	↓*
	Raphe	Adult	↔	↔	↔	↔	↔	ND	↔	↔	ND
		Juvenile	↔	↔	↔	↔	↔	ND	↔	↔	ND
	Hippocampus	Adult	↔	↔	↔	↔	↔	ND	↔	↔	ND
		Juvenile	↑	↔	↔	↔	↔	ND	↔	↔	ND
Protein expression	RN46-B14 cell line		X	X	X	X					
	Raphe	Adult	↔	↔	↔	X					
		Juvenile	↓	↔	↔	X					
	Hippocampus	Adult	↔	ND	ND	↔					
		Juvenile	↔	↔	↔	↑*					

* denotes $P<0.05$ (unpaired t-test)

↑ denotes a trend towards increased expression

↓ denotes a trend towards decreased expression

↔ denotes no change in expression

X denotes expression could not be measured

ND denotes expression not determined

Table 4.3: Summary of all gene and protein changes determined *in vitro* and *in vivo*, following 13-*cis*-RA treatment.

confirmed expression of serotonergic/monoaminergic components in these same brain regions (TPH2, SERT, 5-HT_{1A}R, 5-HT_{1B}R, MAOA, COMT and D2DR), give rise to the possibility that the retinoid signalling pathways may regulate the expression of monoaminergic genes in the rat raphe nuclei (both *in vivo* and *in vitro*) and hippocampus.

4.4.2. Increased D2DR expression

The determination of the gene/protein expression of D2DR acted as a positive control in our experiments given that the D2DR possesses a verified RARE in the promoter region of the gene (Samad *et al.*, 1997). Previous *in vitro* studies have measured an increase in D2DR mRNA expression in response to ATRA treatment (Samad *et al.*, 1997; Sodja *et al.*, 2002; Valdenaire *et al.*, 1998). In addition, in RARβ/RXRβ, RARβ/RXRγ and RXRβ/RXRγ double null mice there is reduced D2DR mRNA expression in the striatum (Krezel *et al.*, 1998). Here, in RN46A-B14 cells, a significant increase in D2DR gene expression was observed after 48 h incubation with 10μM 13-*cis*-RA, whereas RN46A-B14 cells treated with a lower concentration of 13-*cis*-RA (2.5μM) had a trend for increased D2DR mRNA expression. This suggests that at 10μM of 13-*cis*-RA, the cell line had received an appropriate level of retinoids to activate RAR/RXR mediated gene transcription, whereas this is not the case at the lower concentration.

In relation to the raphe, the elevation of D2DR gene expression following 13-*cis*-RA treatment appears to be specific to the *in vitro* cell line, as no similar *in vivo* effects were seen in the adult or juvenile rats. However, 13-*cis*-RA treatment did produce a significant increase in D2DR protein levels in juvenile, but not adult, rat hippocampal tissue. The greater increases of D2DR protein expression in the hippocampus, compared with the raphe, may reflect differences in endogenous D2DR expression. D2DR is highly expressed in the hippocampus (Jaber *et al.*, 1996) but only has low abundance in the raphe (Martin-Ruiz *et al.*, 2001). This may explain our inability to measure the protein levels of D2DR in neither the raphe tissue of rats nor the raphe-derived RN46A-B14 cell line. An alternative explanation is that retinoid receptor numbers are greater in hippocampus and therefore retinoid

signalling is more prominent in this structure compared with the raphe. Although one-step RT-PCR qualitatively confirmed strong expression of all retinoid receptor subtypes in the raphe nuclei, the hippocampus is known to contain high numbers of RARα, RARγ and RXRβ (Krezel *et al.*, 1999).

Numerous studies have shown that D2DR$^{-/-}$ mice have locomotor impairments (Baik *et al.*, 1995; Jung *et al.*, 1999; Kelly *et al.*, 1998), whereas administration of D2-like agonist quinpirole is thought to alter locomotion (Zhou *et al.*, 1991). Therefore the behavioural consequences of elevated D2DR expression, as a result of chronic 13-*cis*-RA treatment, may be to modify locomotion, although other brain regions and pathways are also significantly involved in locomotion (Garcia-Campmany *et al.*, 2010). However, we did not observe changes in rat locomotion as measured by the open field test (Figure 3.11), suggesting that the increases observed in juvenile rat hippocampus D2DR protein expression (Figure 4.14D) was not of a sufficient magnitude to induce an altered locomotor phenotype.

Alternatively, changes in D2DR expression may be involved in the pathology of depression. Most animal studies have shown that D2-like receptor antagonists block the antidepressive action of amitriptyline or desipramine (reviewed in (Gershon *et al.*, 2007), while the D2-like agonist pramipexole was shown to increase sucrose consumption in stressed and non-stressed rats (Willner *et al.*, 1994). Additionally, a myriad of antidepressant drugs increase D2DR and D3DR binding activity in the nucleus accumbens of rodents (Dziedzicka-Wasylewska *et al.*, 2002), while human antidepressant responders show a similar increase in basal ganglia D2DR and D3DR binding activity (Klimke *et al.*, 1999). Therefore, the literature suggests that depression may arise as a result of reduced dopaminergic signalling, whereas increased D2DR signalling is thought to be antidepressive. Therefore our observation that 13-*cis*-RA treatment increases D2DR expression suggests an antidepressive effect rather than a pro-depressive effect. However, it is worth noting that depressed patients have been shown to have a compensatory upregulation of D2DRs in the basal ganglia/cerebellum (D'Haenen H *et al.*, 1994), so it is possible that our observed increase in D2DR similarly reflects a compensatory mechanism in response to a hypo-dopaminergic state.

4.4.3. No change in SERT and 5-HT$_{1A}$R expression

The data from gene expression and protein level studies indicate that 13-*cis*-RA has no effect on SERT or 5-HT$_{1A}$R expression in the raphe nuclei and hippocampus of juvenile and adult rats, as well as the RN46A-B14 cell line (Table 4.3). The study of the RN46-B14 cell line closely mirrored previous work within our group (O'Reilly *et al.*, 2007) whereby the gene expression/protein levels of SERT and 5-HT$_{1A}$R were determined, and while we similarly observed no change in mRNA expression (Figure 4.7), we were not able to replicate the finding of increased protein levels of SERT and 5-HT$_{1A}$R due to methodological difficulties (Chapter 4.3.3.1.). However, the analysis of the gene expression and protein levels of SERT and 5-HT$_{1A}$R in both the juvenile and adult rat raphe nuclei revealed 13-*cis*-RA treatment had no significant effects (Figure 4.10 and Figure 4.13, respectively). It therefore suggests that the previously observed increases in SERT and 5-HT$_{1A}$R protein levels *in vitro* following 13-*cis*-RA treatment are limited to this cell line and do not occur in the rat raphe nuclei.

The lack of altered protein levels of SERT and 5-HT$_{1A}$R in the raphe nuclei and hippocampus indicates that the functional impact of 13-*cis*-RA in serotonergic pathways may be limited and has a profound effect on the hypothesis of retinoid-induced depression. SERT is critical in the homeostasis of synaptic 5-HT given that SERT$^{-/-}$ mice possess a six-fold increase in extracellular 5-HT (Fabre *et al.*, 2000; Mathews *et al.*, 2004), selective blockade of SERT via SSRIs increases extracellular 5-HT in the raphe nuclei (Bel *et al.*, 1992; Gartside *et al.*, 1995) and mice that have a two- to threefold overexpression of SERT have reduced extracellular levels of 5-HT (Jennings *et al.*, 2006). Therefore, an increase in SERT protein expression in the raphe nuclei would be expected to lead to greater re-uptake of extracellular 5-HT back to the presynaptic terminal and induce a hyposerotonergic state and this process could underlie the increased susceptibility to depression following 13-*cis*-RA treatment. Although I have shown that SERT protein levels remain unaltered by 13-*cis*-RA, it is conceivable that changes may occur at the plasma/tissue level and not necessarily at the extracellular level, given that changes in plasma and tissue 5-HT levels have also been shown in SERT$^{-/-}$ and overexpressing SERT mice (Fabre *et al.*, 2000; Jennings *et al.*, 2006). This is investigated and discussed in Chapter 5.

Meanwhile, the precise role and association between SERT and depression is unclear, although conversely, the ability of SSRIs to alleviate depression through the blockade of SERT is well established. For instance, some post-mortem studies of depressed suicide victims have shown an increase in SERT numbers in the frontal cortex, hippocampus, thalamus and striatum (Arato *et al.*, 1991; Cannon *et al.*, 2007; Gross-Isseroff *et al.*, 1989), although no change in SERT-specific radioligand binding has been recorded in the raphe nuclei of depressed suicide victims (Arango *et al.*, 2001; Bligh-Glover *et al.*, 2000; Cannon *et al.*, 2007). There is disputably more evidence that SERT-specific radioligand binding is *reduced* in depressed suicide victims (reviewed in (Purselle *et al.*, 2003)). Moreover, $SERT^{-/-}$ mice exhibit depression-like behaviour (Holmes *et al.*, 2003; Lira *et al.*, 2003) such as increased immobility in the FST (Holmes *et al.*, 2003; Olivier *et al.*, 2008) and increased immobility in the tail suspension test (Alexandre *et al.*, 2006), whereas SERT overexpressing mice appear to have a low-anxiety phenotype (Jennings *et al.*, 2006). The paradoxical depressive behaviour observed in $SERT^{-/-}$ mice may relate to reductions in serotonergic cell number in the DRN and firing rate, as opposed to changes in extracellular 5-HT levels (Lira *et al.*, 2003). It is therefore unclear as to the definitive role that SERT plays in depression pathology and therefore makes it difficult to determine whether increases in SERT mRNA/protein following 13-*cis*-RA treatment would be likely to be pro-depressive in any case.

The inability of 13-*cis*-RA to affect -HT_{1A}R protein levels is equally as significant. 5-HT_{1A}Rs in the DRN are known to reduce the firing rate of these neurons, the 5-HT released and 5-HT synthesis (Blier *et al.*, 1987; Hjorth *et al.*, 1991; Hutson *et al.*, 1989; Kreiss *et al.*, 1994; Meller *et al.*, 1990; Sprouse *et al.*, 1986; Verge *et al.*, 1985; Wang *et al.*, 1977). Therefore, an increase in the 5-HT_{1A}R population induced by 13-*cis*-RA treatment in the rat raphe nuclei would be expected to lead to further reductions of firing, 5-HT release and synthesis and would result in a hyposerotonergic state that may mediate depression. Conversely, a decrease in 5-HT_{1A}R protein levels induced by 13-*cis*-RA in the rat raphe nuclei would increase firing rate, 5-HT release and 5-HT synthesis and result in a hyperserotonergic state that would appear unlikely to mediate depression. In summary, two vital

components of serotonergic signalling and regulation are not affected by 13-*cis*-RA treatment, although the possibility remains that other serotonergic components may be altered.

4.4.4. Trends for altered TPH2 expression

TPH2 gene expression remained largely unaltered by 13-*cis*-RA treatment in the rat raphe nuclei, rat hippocampus and RN46A-B14 cell line. However, a trend for increased TPH2 gene expression was evident in the juvenile hippocampus, although not evident at the corresponding protein level. In contrast, Western blot analysis revealed a trend for a decrease in TPH2 protein levels in the juvenile rat raphe nuclei (although a corresponding change in TPH2 gene expression was not found, Table 4.3). These findings suggest that TPH2 gene expression and TPH2 protein levels may be dissociable.

The central dogma of molecular biology suggests a strong correlation between gene and protein expression (Crick, 1970), although past empirical studies suggest only a modest correlation exists (Nie *et al.*, 2007) such that the use of gene expression patterns may be insufficient to predict the abundance of proteins (Chen *et al.*, 2002; Cox *et al.*, 2005). The reasons behind this discrepancy may lie with additional post-transcriptional mechanisms, post-translational modifications and timing differences between gene and protein expressions (Waters *et al.*, 2006). Some of the post-transcriptional mechanisms include the different efficiencies with which mRNA may be translated into polypeptides (Mata *et al.*, 2005) and the alternative splicing of mRNA that creates different proteins with different cellular locations (Black, 2003). Pertinently, ATRA treatment has been shown to have post-translational effects by increasing Pre-B cell leukaemia transcription factor protein stability in P19 cells (Qin *et al.*, 2004), although there is currently no evidence that ATRA treatment is able to decrease protein stability. Therefore, it is possible that 13-*cis*-RA treatment may increase mRNA expression via RAR/RXR mediated increases in transcription in the juvenile hippocampus, and decrease TPH2 protein levels in the juvenile rat raphe nuclei through non-genomic effects on protein stability. Alternatively, the trend for increased TPH2 gene expression may have occurred due to increased TPH2 mRNA stability, as previous studies have shown that ATRA increases calbindin-

D28 (Wang *et al.*, 1995), acetylcholine esterase (Coleman *et al.*, 1996) and keratin 19 (Crowe, 1993) mRNA stability.

The functional consequences of potentially reduced TPH2 levels have been highlighted by a number of studies. $TPH2^{-/-}$ mice have been shown to display a 96% reduction in 5-HT levels in the dorsal raphe (Alenina *et al.*, 2009), whereas another study found that $TPH2^{-/-}$ mice have a 95% reduction in brain stem 5-HT levels and increased depression-related behaviour (Savelieva *et al.*, 2008). Meanwhile, the TPH2 SNP, G1463A, has been found in depressed patients and results in an 80% loss of production of 5-HT synthesis in PC12 cells (Zhang *et al.*, 2005). A similar murine SNP, C1473G, leads to decreased 5-HT levels in PC12 cells, while BALB/cJ and DBA/2 mice that are homozygous for the 1473G allele have reduced brain 5-HT tissue content/synthesis compared with C57Bl/6 and 129X1/SvJ mice that are homozygous for the 1473C allele (Zhang *et al.*, 2004). Knockin mice with the TPH2 polymorphism R441H, found in a small group of depressed patients, have an 80% reduction in TPH2 enzyme activity and reduced 5-HT levels in the striatum, frontal cortex and hippocampus, along with an increase in depression-related behaviour (Beaulieu *et al.*, 2008). In the context of our hypothesis, the ability of 13-*cis*-RA to reduce TPH2 expression would in turn reduce 5-HT levels in the brain, potentially leading to a hyposerotonergic state and an increased susceptibility to depression.

Meanwhile, it is possible that the counterintuitive increases of TPH2 at the mRNA level in the juvenile rat hippocampus, in direct contrast to the lack of alterations at the protein level, represent a secondary compensatory response to counteract reduced 5-HT levels and therefore reverse the hyposerotonergic state of this brain region. The trend for increased TPH2 mRNA is similar to other reports of increased TPH2 mRNA in depressed suicides (Bach-Mizrachi *et al.*, 2006). The reasons underpinning this paradoxical increase in TPH2 mRNA may be a homeostatic response to low 5-HT levels and/or reduced expression of TPH2 protein, although further investigation is required.

4.4.5. Lack of 13-*cis*-RA-induced changes in other genes of interest

When analysing the second positive control, RARα, I found 13-*cis*-RA treatment had no effect on its gene expression *in vitro* and in both the adult/juvenile rat tissues. It was somewhat surprising to detect significant increases in D2DR (*in vitro* and in the juvenile rat hippocampus) without changes in RARα gene expression, given that they both contain verified RAREs (Brand *et al.*, 1988; Petkovich *et al.*, 1987). It remains possible that the upregulation of RARα by 13-*cis*-RA is small in comparison to D2DR and/or was masked by the small sample size. If RARα expression was found to be increased by 13-*cis*-RA treatment, it could indicate a positive feedback system whereby 13-*cis*-RA increased the expression of retinoid receptors, making the brain increasingly more sensitive to further retinoid treatment. In addition to RARα, we analysed RARβ gene expression in RN46A-B14 cells and had hypothesized an upregulation in response to 13-*cis*-RA treatment, although our data found a significant decrease. The reasons behind this are unclear, as we had expected this subtype of retinoid receptor to act in a similar fashion to RARα (Brand *et al.*, 1988; Lane *et al.*, 2005; Petkovich *et al.*, 1987). It is possible this effect is specific to RN46A-B14 cells and does not represent the normal physiological function of RARβ *in vivo* and was therefore not further utilised in subsequent experiments.

We also analysed the gene expression of monoaminergic components 5-HT_{1B}R and MAOA *in vitro* and in the raphe nuclei/hippocampus of chronically treated rats. Overall, 13-*cis*-RA was not found to alter the expression of these genes in the raphe nuclei, hippocampus or RN46A-B14 cell line, although 5-HT_{1B}R may be a notable exception given that 5-HT_{1B}R gene expression is elevated in the RN46A-B14 cell line. Increased levels of MAOA might have suggested a greater rate of 5-HT and DA metabolism in the synaptic cleft that could cause a hyposerotonergic and hypodopaminergic state. The consequence of altered 5-HT_{1B}Rs is unclear given that their role in depression pathology is not completely understood. For instance, the frequency of two 5-HT_{1B}R polymorphisms (G861C and C129T) in patients with a history of major depression was shown to be not significantly different from controls (Huang *et al.*, 1999) and these findings appear to be corroborated by similar studies

conducted in suicide victims (New *et al.*, 2001; Nishiguchi *et al.*, 2001). The same study also found no association between major depression and the binding indices of the 5-HT_{1B}R in the prefrontal cortex, although an earlier study had indicated a decrease in the binding affinity of 5-HT_{1B}R in depressed suicides (Arranz *et al.*, 1994).

Overall, these findings, in conjunction with those described in Chapter 4.4.3., suggest a lack of direct evidence to support the association between 13-*cis*-RA treatment and alterations in monoaminergic components (5-HT_{1A}R, SERT, 5-HT_{1B}R and MAOA). However, the possibility exists that *i)* serotonergic pathways may be altered through TPH2 and were not adequately detected in my studies, *ii)* other untested serotonergic components may be involved, *iii)* dopaminergic pathways are altered as evidenced by increased D2DR protein levels *in vitro* and *in vivo*, *iv)* serotonergic and dopaminergic pathways are both implicated and influence one another or *v)* an entirely different system that is known to mediate depression, such as neurogenesis, is responsible for 13-*cis*-RA-induced depression.

4.4.6. Putative sensitivity of juvenile rats to 13-*cis*-RA-induced molecular alterations

The significant increase in D2DR protein levels in the rat hippocampus and diametric trends in altered TPH2 gene expression and protein levels (in hippocampus and raphe nuclei, respectively), all derive from juvenile 13-*cis*-RA-treated rats as opposed to the equivalent adult rats. Differences in the endogenous levels of TPH2 in juvenile and adult rats appear to be an unlikely explanation as a previous study has shown that the expression of TPH2 mRNA in one month old mice does not differ from 2 month and 4 month old mice (Gutknecht *et al.*, 2009). Meanwhile, there is some evidence of ontogenetic differences in D2-like receptor numbers, with around a third to a half of receptors being lost from adolescence to adulthood in the human striatum (Seeman *et al.*, 1987a) and are thought to peak at postnatal day 40 in the rat striatum and gradually decline in adult rats (Tarazi *et al.*, 1999; Tarazi *et al.*, 1998). However, such findings have not been universally found in humans (Palacios *et al.*, 1988), while no age-related changes in D2-like receptor numbers were observed in the prefrontal

cortex and hippocampus (Tarazi *et al.*, 1999; Tarazi *et al.*, 1998). The data therefore raises the possibility that the molecular targets of 13-*cis*-RA are more susceptible to alteration in juvenile rats and less so in adult rats. This would suggest that juvenile rats are particularly susceptible to 13-*cis*-RA-induced molecular alterations, which in turn, might predispose this age-group to the pro-depressive effects of 13-*cis*-RA. It is currently not known if differences exist in the protein levels of TPH2 amongst juvenile and adult depressive patients, although it is becoming increasingly clear that there are molecular differences between both depressed age groups (Kaufman *et al.*, 2001).

4.4.7. Limitations of the gene expression and protein level studies

There are number of limitations with the studies conducted in this chapter to assess 13-*cis*-RA-induced gene expression and protein level alterations. Perhaps the most pertinent, is the small group numbers used in both real-time RT-PCR analysis and densitometric analysis of Western blots. This greatly reduces the statistical power with which to detect significant changes, particularly when a wide degree of variance exists within the data. This is further compounded by expressing the effects of 13-*cis*-RA as relative to vehicle-treated rats, with the latter group also possessing some large degree of variation in the expression of genes and/or levels of protein.

In the real-time RT-PCR analysis of gene changes, it was somewhat surprising to observe large differences in rRNA C_T values, as they should remain fairly consistent both within vehicle and 13-*cis*-RA-treated groups but also across the treatment groups (rRNA expression is highly ubiquitous and not thought to be affected by treatment, therefore making it a frequently used housekeeper gene). This may represent a methodological error, whereby there was an inability to accurately replicate the data from one animal to the next, perhaps due to different quantities of cDNA used. Similar differences were evident for the C_T values of the GOI, within vehicle and 13-*cis*-RA treatment groups, although this may simply represent inherent individual variations in the expression these genes or may also derive from experimental error.

The ensuing result of large standard deviations is particularly pronounced in the gene expression study of the adult raphe (Figure 4.10A). The $2^{-\Delta\Delta Ct}$ method of analysis requires the compairons of the C_T values of the GOI in 13-*cis*-RA treated animals (relative to the rRNA C_T values of these animals) compared against the C_T values of the GOI in vehicle-treated animals (relative to the rRNA C_T values of these animals). The averages of all four sets of data required in this comparison are used and the overall standard deviation is calculated (total standard error= $\sqrt{}$ [(standard deviation 1)^2 + (standard deviation 2)^2 +...]), thereby increasing the standard deviation. This simply calculates the standard deviation for ΔΔCt and so the standard deviation is therefore exponentiated to the base two, to obtain the standard deviation for $2^{-\Delta\Delta Ct}$. Therefore, this analysis is highly susceptible to large standard deviations and subsequent standard errors (unless C_T values are highly consistent) and makes analysing the gene expression data from the adult raphe particularly difficult. Future studies may look to repeating these studies, with greater group numbers (determined by power analysis), such that statistically significant effects may become apparent.

4.4.8. Conclusion

Overall, 13-*cis*-RA treatment was shown to significantly increase D2DR gene expression in the RN46A-B14 cell line and increase D2DR protein expression in the juvenile rat hippocampus, along with a surprising decrease in RARβ gene expression in the RN46A-B14 cell line. However, there is no overwhelming link between 13-*cis*-RA treatment and altered serotonergic pathways, with the exception of a trend for TPH2 protein levels to be reduced in the juvenile rat raphe nuclei and increased gene expression in the juvenile hippocampus. Interestingly, juvenile rats may be susceptible to molecular alterations following 13-*cis*-RA treatment, although this does not appear to result in divergent behaviour, given that 13-*cis*-RA treatment did produce age-related behavioural effects (Chapter 3).

In partial agreement with the original hypothesis, the data suggests dopaminergic pathways are sensitive to 13-*cis*-RA treatment, and specifically, in the hippocampus, although it is unclear from the

literature how altered D2DR protein levels may underlie pro-depressive behaviour. The inability for 13-*cis*-RA treatment to alter serotonergic gene expression or protein levels in the raphe nuclei suggest this brain region and pathway may not be involved in the pro-depressive effects of 13-*cis*-RA, although changes in TPH2 may be significant with greater group numbers. If the effects of 13-*cis*-RA on TPH2 are subsequently found to be significant, a reduction in TPH2 protein in the raphe nuclei could have important functional implications: levels of 5-HT synthesis might be reduced, which in turn could reduce 5-HT release and lead to a reduction in extracellular 5-HT. The potential reduction of extracellular 5-HT might account for the depressive symptoms reported in 13-*cis*-RA patients (discussed in further detail in Chapter 6.4.). However, early research into 5-HT levels in depressed patients alluded to the reduction of 5-HT levels in the CSF, brain tissue and plasma (see Chapter 1.3.1.1.), suggesting a hyposereotonergic state that was not limited to an extracellular location. Similarly, lower levels of DA and/or DA metabolites have been found in the serum and CSF of depressed patients (Engstrom *et al.*, 1999) that might suggest a hypo-dopaminergic state in depression. To investigate whether 13-*cis*-RA-induced alterations exist in the serotonergic or dopaminergic pathways, 5-HT and DA levels were assessed in the raphe nuclei, hippocampus and prefrontal cortex tissue of 13-*cis*-RA-treated animals (along with NA and 5-HT metabolite 5-HIAA), in addition to 5-HT and 5-HIAA levels in the plasma of treated animals, using HPLC analysis (described in Chapter 5).

Chapter 5

The changes in monoamine levels of brain tissue and plasma mediated by 13-*cis*-RA administration

5.1 Introduction

Our original hypothesis asserted that 13-*cis*-RA, acting via retinoid receptors (see Figure 1.2), could alter the expression of genes thought to be involved in the pathology of depression. In turn, the alterations of depression-related genes would be reflected in alterations at the protein level and may result in functional changes. 13-*Cis*-RA treatment *in vivo* lead to a significant increase in D2DR protein levels in the hippocampus, suggesting that the dopaminergic system may be amenable to 13-*cis*-RA-induced alterations, particularly in the hippocampus, although it is currently unknown what effect D2DR has on DA levels. We therefore sought to assess DA levels in the hippocampal tissue of 13-*cis*-RA-treated animals, in addition to analysing DA in the raphe nuclei and prefrontal cortex tissue. 13-*Cis*-RA treatment *in vivo* may lead to a reduction in TPH2 protein levels in the raphe nuclei (Chapter 4), although more work is required to substantiate this. Previous studies have shown that the homozygous knockout of TPH2 leads to an almost total ablation of 5-HT in the brain (Alenina *et al.*, 2009; Savelieva *et al.*, 2008), whereas in heterozygous animals with reduced TPH2 expression there is no effect on 5-HT (Alenina *et al.*, 2009). We have therefore sought to determine whether a possible reduction in TPH2 protein levels in 13-*cis*-RA-treated adult rats may manifest as a significant reduction in brain tissue and plasma levels of 5-HT and its metabolite 5-HIAA, using high performance liquid chromatography (HPLC). Moreover, we have studied whether 13-*cis*-RA treatment has any on NA brain tissue levels, given that this monoamine pathway is similarly thought to be implicated in depression pathology (see Chapter 1.3.1.3.).

Numerous lines of evidence suggest that depression is characterised by low levels of the monoamines 5-HT, DA and NA (reviewed in (Lanni *et al.*, 2009)). The evidence of reduced tissue levels of 5-HT in depression was first shown in post-mortem studies of the hind-brain of depressed suicide patients (Bourne *et al.*, 1968; Shaw *et al.*, 1967) and subsequently in the whole brain, hypothalamus and amygdala of depressed patients (Csernansky *et al.*, 1993; Tuinier *et al.*, 1995). Other indirect measurements of reduced 5-HT levels in depressed patients have come from the measurement of low 5-HT in blood platelets (Coppen *et al.*, 1978) and decreased tryptophan in the blood plasma (Coppen

et al., 1973; Cowen *et al.*, 1989). Moreover, acute dietary tryptophan depletion is known to cause a transient but pronounced return of depressive symptomatology in patients previously treated with antidepressants (Bell *et al.*, 2001; Smith *et al.*, 1997). Additionally, studies have shown that depressed patients have reduced levels of the 5-HT metabolite 5-HIAA in the CSF (Asberg *et al.*, 1976; Reddy *et al.*, 1992). The measurement of 5-HIAA in the CSF is thought to reflect the serotonergic activities of the brain (Stanley *et al.*, 1985) and has also been utilised as an indication of the rate of 5-HT metabolism or 5-HT turnover when used as a ratio with 5-HT (Moir *et al.*, 1970).

A small number of studies have found an association between reduced NA and DA neurotransmitter levels and depression. The levels of the NA metabolite 3-methoxy-4-hydroxy-phenylglycol has been shown to be reduced in the CSF, plasma and urine of depressed patients, although there is great variability among patients and studies (Maas *et al.*, 1972; Potter *et al.*, 1993; Roy *et al.*, 1986). Furthermore, a more recent study has shown that 3-methoxy-4-hydroxy-phenylglycol levels are reduced in the venoarterial plasma of depressed patients (Lambert *et al.*, 2000). The depletion of NA by the administration of the tyrosine hydroxylase inhibitor α-methyl-para-tyrosine is known to cause a relapse of depressive symptoms in patients who had previously been treated with a NA-reuptake inhibitor (Booij *et al.*, 2003; Ordway *et al.*, 1998), whereas elevated levels of tyrosine hydroxylase have been reported in the locus coeruleus of suicide victims (Ordway *et al.*, 1994; Zhu *et al.*, 1999). Meanwhile, reduced levels of DA in the plasma of depressed patients have been reported (Hamner *et al.*, 1996), along with reduced levels of the DA metabolite homovanillic acid (HVA) in the CSF of suicide attempters and depressed subjects (Engstrom *et al.*, 1999; Jones *et al.*, 1990). In fact, the reduction of HVA is thought to be one of the most consistent findings when comparing monoamine metabolites in the CSF of depressed patients and healthy volunteers (Goodwin *et al.*, 1990).

Despite the involvement of retinoids in the regulation of neuronal gene transcription, it is unclear as to whether retinoids are able to have direct effects on monoamine levels in the brain. One study using vitamin A deficient rats (3 wks old at start of deficient diet), demonstrated no significant differences in the accumulation of the DA precursor 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum

(Carta *et al.*, 2006). However, the striatal content of acetylcholine was reduced significantly in vitamin A deficient rats and suggests that retinoids are required for maintaining acetylcholine levels in the adult brain. In another study, the administration of 3mg of retinol to newborn female rats led to a reduction of brain tissue levels of 5-HIAA at 3 months of age and similarly affected the untreated progenies of these female rats (Tekes *et al.*, 2009a; Tekes *et al.*, 2009b). The treated adult rats and their adult untreated progenies displayed reduced levels of 5-HIAA in the frontal cortex, hypothalamus and hippocampus compared with controls, increased levels of 5-HIAA in the striatum, increased levels of NA in the brainstem, increased levels of DA in the striatum and decreased levels of HVA in the frontal cortex. Although these studies observed alterations in monoamine levels in the adult brain, the manipulations to the retinoid system were undertaken neonatally and may therefore highlight developmental disturbances rather than a direct link between retinoid signalling and monoamine levels in the adult brain.

The association between 13-*cis*-RA treatment and monoamine levels in the adult brain is largely unknown, with the exception of one study (Ferguson *et al.*, 2005b). In male and female rats that received 7.5 and 15mg/kg of 13-*cis*-RA for 28 days, there were no effects on DA, DOPAC, HVA, 5-HT or 5-HIAA concentrations in the frontal cortex, hippocampus or diencephalon although some differences in the striatal tissue were reported (Ferguson *et al.*, 2005b). The study conducted by Ferguson did not look at the raphe nuclei region; a site that is rich in 5-HT cell bodies, the location of 5-HT synthesis and a region that has been proposed to have a role in depression pathology. We have therefore investigated whether the chronic treatment of 13-*cis*-RA in adult rats would be sufficient to alter the monoamine levels of 5-HT and the metabolite 5-HIAA within the raphe nuclei. Furthermore, 5-HT and 5-HIAA levels were assessed in the hippocampal and prefrontal cortex tissue, as well as tissue levels of DA, HVA and NA in the same brain regions.

Plasma 5-HT levels are thought to reflect those in the CSF (Sarrias *et al.*, 1990), while platelets share a number of characteristics with serotonergic neurons in the CNS (Da Prada *et al.*, 1988; Owens *et al.*, 1994). For example, platelets and serotonergic neurons both express SERT and demonstrate active

uptake of 5-HT (Pletscher, 1987). Depressed patients are known to have diminished 5-HT uptake in platelets (Coppen *et al.*, 1978), low tryptophan levels in the plasma (Coppen *et al.*, 1973; Cowen *et al.*, 1989), reduced binding of imipramine to platelets (Mossner *et al.*, 2007) and increased numbers of 5-HT_{2A}R (Mendelson, 2000). Therefore, we have also investigated whether chronic 13-*cis*-RA treatment altered adult rat peripheral levels of 5-HT and the metabolite 5-HIAA in platelet-rich plasma (PRP), which is defined as having a platelet count two- to seven-fold of blood (Marx *et al.*, 1998; Pietrzak *et al.*, 2005; Weibrich *et al.*, 2002), and platelet-poor plasma (PPP) which is deficient in platelets.

5.2 Methods

5.2.1 HPLC analysis of brain tissue samples

Adult rats first underwent 6 weeks of vehicle or 13-*cis*-RA treatment, followed by the study of 8-OH-DPAT-induced hypothermia (described in Chapter 3.2.6.). Following a two day washout period, whereby only 13-*cis*-RA was administered and not 8-OH-DPAT or WAY-100635, adult rat brains were removed and the prefrontal cortex, hippocampus and raphe nuclei brain regions were dissected freehand on ice (as described in Chapter 2.2.2.). Dissected tissue samples were snap frozen in liquid nitrogen and stored at -80°C. Each dissected brain region from each rat was weighed (low interindividual variability for each brain region) and then homogenized in 10 volumes of 0.1M perchloric acid containing 100µM ascorbate in a 2ml glass homogenizer (or eppendorf if the total volume was less than 400µl). Precipitated protein was removed by centrifuging at 12,000g for 3 min and the supernatant was kept either as a concentrated sample or diluted 1:5 and 1:10 in 0.1M perchloric acid with 100µM ascorbate. Samples were then stored at -80°C before analysis.

Levels of 5-HT, 5-HIAA, DA, HVA and NA were determined by HPLC with electrochemical detection. The concentrated or diluted 1:10 samples (depending on peak height) were loaded individually into an autosampler (50µl, Perkin Elmer series 200 autosampler) and the monoamines and metabolites were separated using a mobile phase (0.1M sodium dihydrogen orthophosphate, 2.5mM octylsulphonate, 0.5mM EDTA, 1.025% acetic acid, 12% methanol, pH 3.0) at a flow rate of 1ml/min (Jasco pump, PU-1580) through the column (Column- Supelcosil LC-18, 4.6 mm id x 15 cm, 3µM particle size). Electrochemical detection was achieved with +0.7V applied across the cell (Decade, Antec Leyden, 50µM spacer) and a range of 50nA/10V. The samples were identified by comparison of their retention time to standard solutions of each monoamine and metabolite compound (50µl of 50ng/ml for 5-HT, DA, HVA, NA and 5ng/ml for 5-HIAA, made in 0.1M perchloric acid and 100µM ascorbate). The monoamine and metabolite levels were calculated by comparing the peak areas obtained from the samples to the peak areas obtained with the standard solutions (all standards obtained from Sigma, peak areas calculated by PowerChrom v2.2 software, ADI instruments). These

values were subsequently adjusted for the dilution factor used and the volume of 0.1M PCA with 100µM ascorbate added to the tissue, to give the concentration of monoamines and metabolites in nanograms per gram of wet weight tissue.

5.2.2. HPLC analysis of blood plasma samples

Following 6 weeks of vehicle or 13-*cis*-RA treatment, adult rat blood was rapidly collected in eppendorf tubes containing 50µl of K_2EDTA (60mg/ml). The blood samples were gently mixed by inversion and immediately placed on ice. This was followed by centrifugation of the samples at 700*g* for 5 min (4°C). Half of the volume of supernatant, containing PRP, was subsequently removed. The remaining half of PRP underwent further centrifugation at 350*g* for 20 min (4°C) and the supernatant, containing PPP was collected. Both PRP and PPP samples were stored at -80°C before analysis. The PRP and PPP samples (200µl) were added to 0.4M Perchloric acid (200µl), vortexed thoroughly and incubated on ice for 30 min. The samples were subsequently centrifuged at 24,000*g* for 15 min (4°C) and the supernatant was collected.

Levels of 5-HT and 5-HIAA were determined by HPLC with electrochemical detection. The samples were loaded individually into an autosampler (50µl, Perkin Elmer series 200 autosampler) and the monoamines and metabolite were separated using a mobile phase (75mM sodium dihydrogen orthophosphate, 0.3mM octylsulphonic acid, 0.1mM EDTA, 2mM KCl, 17% Methanol, pH 4.0) at a flow rate of 1ml/min (Jasco pump, PU-1580) through the column (Column- Supelcosil LC-18, 4.6mm id x 15cm, 3µM particle size). Electrochemical detection was achieved with +0.51V applied across the cell (Decade, Antec Leyden, 50µM spacer). The samples were identified by comparison of their retention time to standard solutions of each monoamine and metabolite compound (50µl of 20nM for 5-HT and 5-HIAA, made in 0.4M perchloric acid). The monoamine and metabolite levels were calculated by comparing the peak areas obtained from the samples to the peak areas obtained with the standard solutions (peak areas calculated by PowerChrom v2.2 software, ADI instruments) and these values were given in nM. All HPLC experiments were performed with Dr. Cheney Drew.

5.3. Results

5.3.1. The monoamine and metabolite content of the raphe nuclei, hippocampus and prefrontal cortex tissue following 13-*cis*-RA treatment

Initially, standards for each monoamine and metabolite (50µl of 50ng/ml 5-HT, 5-HIAA, DA, HVA and NA) were injected onto the HPLC column. Figure 5.1A shows a chromatogram whereby the standards were eluted with the following retention times: NA=3.21 min (50ng/ml), 5-HIAA=5.04 min (5ng/ml), DA=7.31 min (50ng/ml), HVA=8.59 min (50ng/ml) and 5-HT=16.29 min (50ng/ml).The retention times were found to be highly disparate and reproducible and therefore the peak area of each monoamine and metabolite was easily identified and measured. Similarly, the height of each monoamine and metabolite peak was required to lie within a range from 0 to 1V, as off-scale peaks would prevent the correct determination of monoamine and metabolite concentration. The total length of the experimental running time was 20 min, to allow for the full separation and elution of monoaminergic and metabolite compounds in all subsequent brain tissue samples. All monoamine and metabolite compounds were detected reliably in the raphe nuclei, hippocampus and prefrontal cortex with the exception of HVA, which was frequently represented by a small peak height and peak area that could not be accurately determined above the background reading. Representative chromatograms for the elution of 5-HT, 5-HIAA, DA and NA in adult rat raphe nuclei, hippocampus and prefrontal cortex tissue samples are shown in Figure 5.1.

The HPLC analyses of monoamine and metabolite levels in all of the tissues studied is shown in Figure 5.2 and Table 5.1. 13-*Cis*-RA treatment had no effect on the monoamine and metabolite concentration levels measured in the adult rat raphe nuclei tissue ($P>0.05$, unpaired t-test, Figure 5.2A). Statistical analysis revealed no significant difference between the vehicle and 13-*cis*-RA-treated hippocampal samples for all monoamine and metabolite compounds measured ($P>0.05$, unpaired t-test, Figure, 5.2B). Meanwhile, statistical analysis revealed no significant difference between the vehicle and 13-*cis*-RA-treated prefrontal cortex samples for all monoamine and

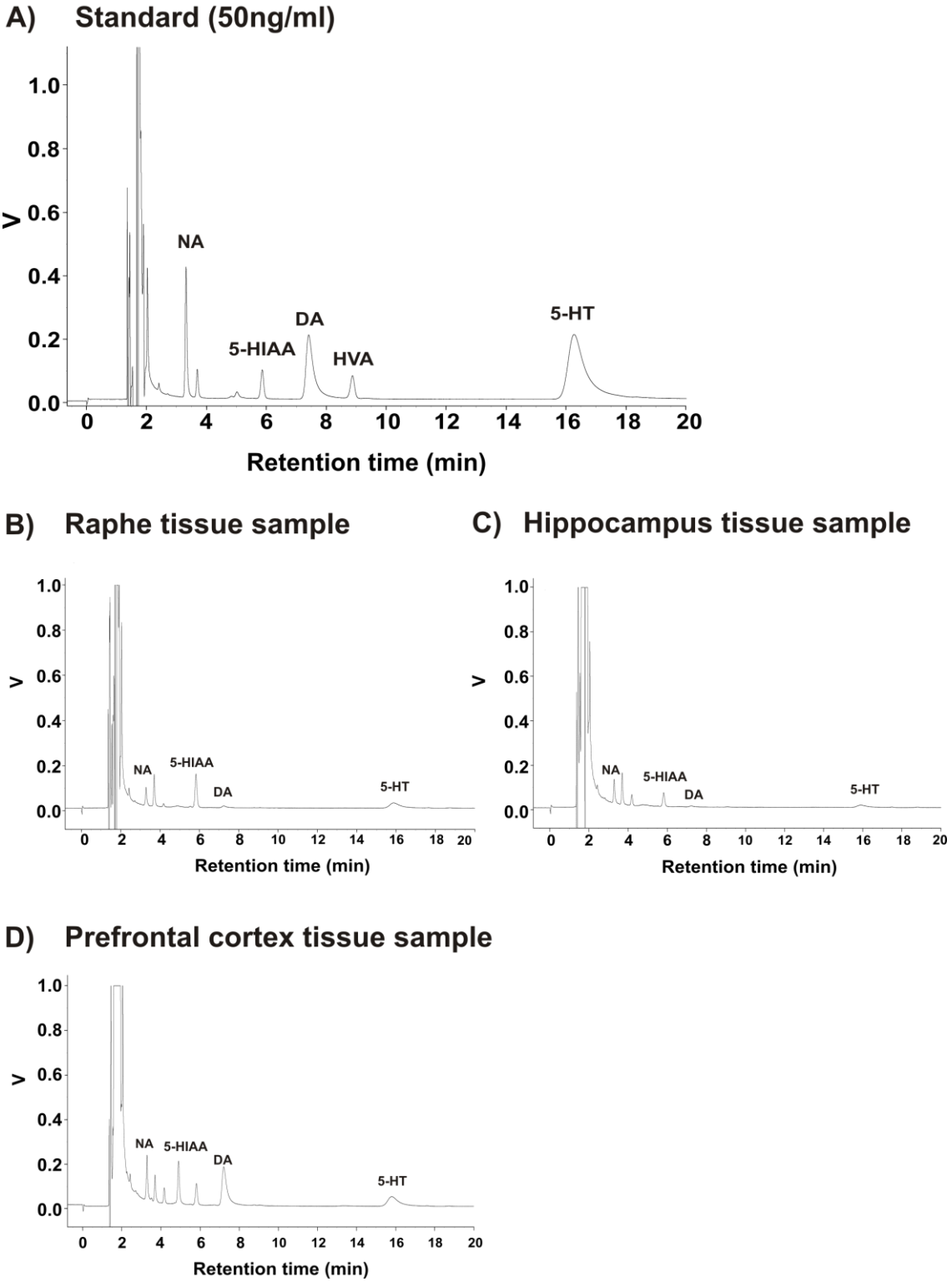

Figure 5.1: Representative HPLC chromatograms of monoamines and metabolite in rat brain tissue. A) Standard solutions for each monoamine and metabolite compound were measured via HPLC (50µl of 50ng/ml for 5-HT, DA, HVA and NA, 5ng/ml for 5-HIAA) and retention times were determined. Adult rat raphe nuclei (B), hippocampus (C) and prefrontal cortex (D) chromatograms showing the eluted monoamine and metabolite peaks (excluding HVA).

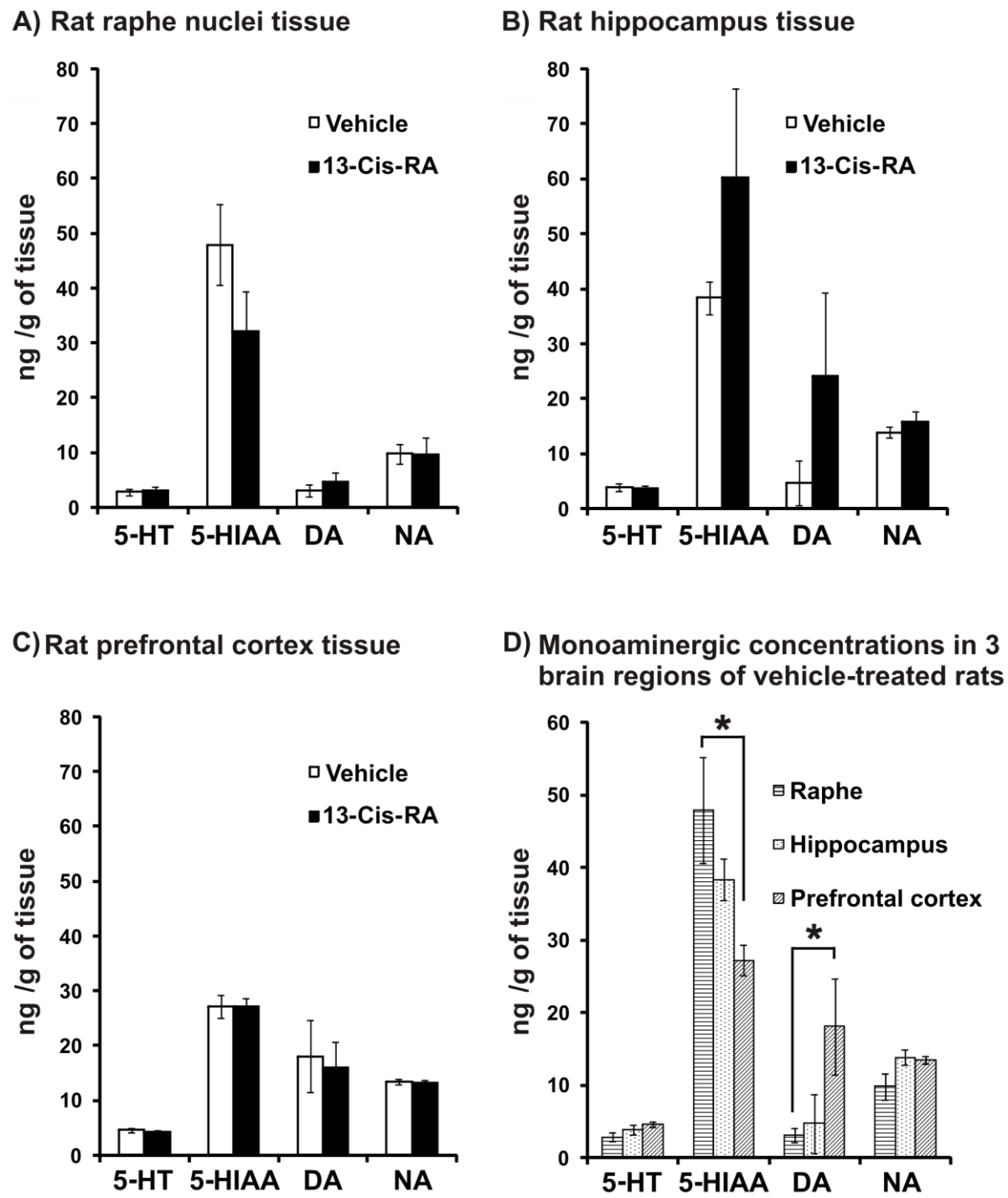

Figure 5.2: Concentration of 5-HT, 5-HIAA, DA and NA in adult rat raphe nuclei (A), hippocampus (B) and prefrontal cortex (C) tissue samples following chronic 13-*cis*-RA treatment. The concentration of 5-HT, 5-HIAA, DA and NA was measured via HPLC in A) the raphe nuclei of vehicle (n=9) and 13-*cis*-RA-treated (n=10-12) adult rats, B) the hippocampus of vehicle (n=8-9) and 13-*cis*-RA-treated (n=12) adult rats and C) the prefrontal cortex of vehicle (n=8) and 13-*cis*-RA-treated (n=12) adult rats. D) shows the differing concentrations of each monoamine and metabolite in the three brain regions assessed in vehicle-treated adult rats. Values are mean ± SEM. * denotes $P<0.05$, t-test.

Monoamine/ metabolite	Raphe nuclei Vehicle	Raphe nuclei 13-*Cis*-RA	Hippo-campus Vehicle	Hippo-campus 13-*Cis*-RA	Prefrontal cortex Vehicle	Prefrontal cortex 13-*Cis*-RA
5-HT	2.85 ± 0.58 (9)	3.18 ± 0.63 (12)	3.91 ± 0.67 (9)	3.66 ± 0.53 (12)	4.59 ± 0.42 (8)	4.19 ± 0.48 (12)
5-HIAA	47.94 ± 7.29 (9)	32.19 ± 7.22 (11)	38.33 ± 2.90 (9)	60.34 ± 16.14 (12)	27.20 ± 2.14 (8)	27.18 ± 1.50 (12)
DA	3.07 ± 1.01 (9)	4.76 ± 1.62 (12)	4.68 ± 4.01 (8)	24.08 ± 15.13 (12)	18.05 ± 6.57 (8)	15.97 ± 4.65 (12)
NA	9.79 ± 1.82 (9)	9.69 ± 3.03 (10)	13.83 ± 0.99 (9)	15.76 ± 1.81 (12)	13.48 ± 0.52 (8)	13.27 ± 0.55 (12)

Table 5.1: The concentration of monoamines and metabolites in the raphe nuclei, hippocampus and prefrontal cortex of 13-*cis*-RA-treated adult rats. Brain tissue levels of 5-HT, 5-HIAA, DA and NA were determined by HPLC with electrochemical detection and values are given as ng/g of wet tissue. Values are mean ± SEM. Numbers in brackets denote n numbers of animals.

metabolite compounds measured (P>0.05, unpaired t-test, Figure 5.2C).

Meanwhile, Figure 5.2D shows the concentration of 5-HT, 5-HIAA, DA and NA in raphe nuclei, hippocampus and prefrontal cortex of vehicle-treated adult rats. The levels of 5-HIAA, the main metabolite of 5-HT, was significantly higher in the raphe nuclei compared with the prefrontal cortex (P=0.021, unpaired t-test) and significantly higher in the raphe nuclei compared with the hippocampus (P=0.044, unpaired t-test). However, the profile of 5-HIAA levels across these three brain regions was not reflected when measuring 5-HT concentrations, which were considerably lower than 5-HIAA and were constant among the brain regions studied. Similarly, the levels of NA in all three brain regions did not appear to differ greatly (P>0.05, unpaired t-test). The levels of DA in the prefrontal cortex were significantly higher than the raphe nuclei (P=0.041, unpaired t-test) and there was a trend for higher DA levels in the prefrontal cortex compared with the hippocampus.

5.3.2. The rate of 5-HT turnover in the raphe nuclei, hippocampus and prefrontal cortex tissue following 13-*cis*-RA treatment

The ratio between 5-HIAA concentration and 5-HT concentration, as a measure of 5-HT turnover (Moir *et al.*, 1970), for each brain region studied is shown in Figure 5.3. The 5-HIAA/5-HT ratios for raphe nuclei (n=9), hippocampus (n=7), and prefrontal cortex (n=8) of vehicle-treated rats were 20.35 ± 3.25, 11.83 ± 1.99 and 6.07 ± 0.34, respectively. Meanwhile, the 5-HIAA/5-HT ratios for the raphe nuclei (n=11), hippocampus (n=10) and prefrontal cortex (n=11) of 13-*cis*-RA-treated rats were 13.45 ± 2.09, 21.35 ± 9.04 and 6.09 ± 0.38, respectively. Chronic 13-*cis*-RA treatment does not have a significant effect on the rate of 5-HT turnover in the raphe nuclei, hippocampus or prefrontal cortex (P>0.05, unpaired t-test), although it should be noted that there is a trend for reduced 5-HT turnover in the raphe nuclei following 13-*cis*-RA treatment (P=0.081, unpaired t-test).

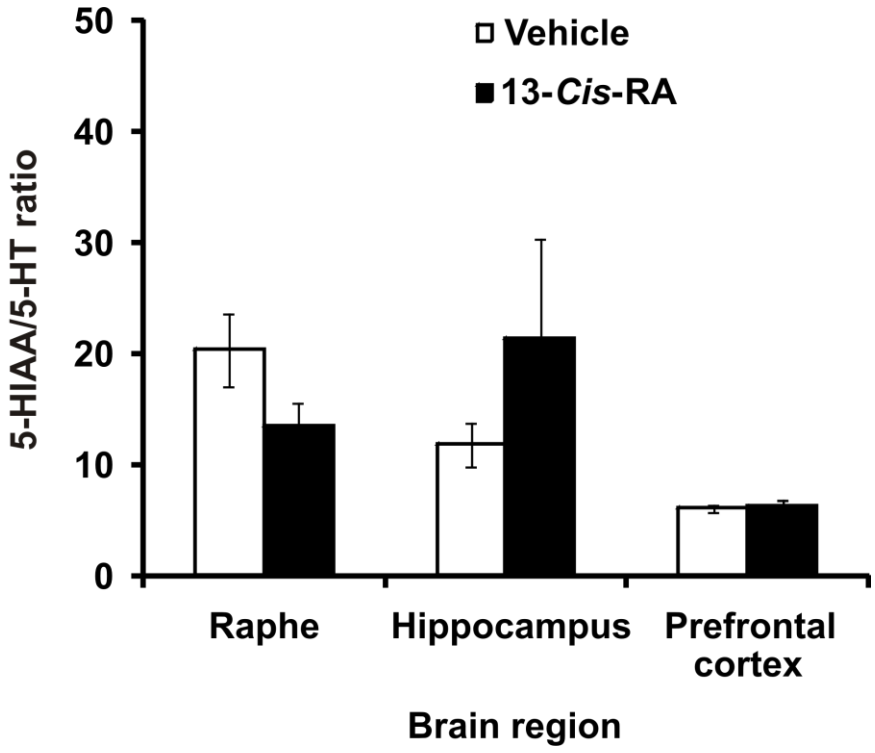

Figure 5.3: 5-HT turnover in the raphe nuclei, hippocampus and prefrontal cortex of 13-*cis*-RA-treated adult rats. The ratio of 5-HIAA/5-HT was calculated in each brain region to give a measure of 5-HT turnover in vehicle-treated (n=7-9) and 13-*cis*-RA-treated (n=10-12) adult rats. Values are mean ± SEM.

5.3.3. The effect of 13-*cis*-RA treatment on 5-HT and 5-HIAA blood plasma levels and 5-HT turnover

Initially, standards for each monoamine and metabolite (50µl of 20nM 5-HT and 5-HIAA) were injected onto the HPLC column. Figure 5.4A shows a chromatogram whereby the standards were eluted with the following retention times: 5-HIAA=4.94 min and 5-HT=6.04 min. The retention times were found to be highly disparate and reproducible and therefore the peak area of each monoamine and metabolite was easily identified and measured. Similarly, the height of each monoamine and metabolite peak was required to lie within a range from 0 to 1V, as off-scale peaks would prevent the correct determination of monoamine and metabolite concentration. The total length of the experimental running time was 14 min, to allow for the full separation and elution of 5-HT and its metabolite in all subsequent PRP and PPP samples. Representative chromatograms for the elution of 5-HT and 5-HIAA in PRP and PPP samples are shown in Figure 5.4B and Figure 5.4C, respectively.

The HPLC analyses of monoamine and metabolite levels in all PRP and PPP samples studied is shown in Figure 5.5 and Table 5.2. 13-*Cis*-RA treatment significantly increased 5-HT levels in the PRP (P=0.03, unpaired t-test), although no such effects were observed in the PPP (P =0.38, unpaired t-test). Statistical analysis revealed no significant difference in 5-HIAA levels between the vehicle and 13-*cis*-RA-treated PRP and PPP samples. Meanwhile, the ratios of platelet 5-HT levels to plasma 5-HT levels in vehicle and 13-*cis*-RA-treated adult rats (shown in Figure 5.5C) reveal that 13-*cis*-RA treatment had no significant effect on 5-HT uptake into the platelets.

The ratio between 5-HIAA concentration and 5-HT concentration, as a measure of 5-HT turnover (Moir *et al.*, 1970), for PRP and PPP is shown in Figure 5.5D. The 5-HIAA/5-HT ratios for PRP (n=12) and PPP (n=12) of vehicle-treated rats were 0.018 ± 0.010 and 0.018 ± 0.011, respectively. Meanwhile, the 5-HIAA/5-HT ratios for the PRP (n=12) and PPP (n=12) of 13-*cis*-RA-treated rats were 0.016 ± 0.001 and 0.030 ± 0.027, respectively. Chronic 13-*cis*-RA treatment did not have a significant effect on the rate of 5-HT turnover in the PRP and PPP of adult rats (P>0.05, t-test).

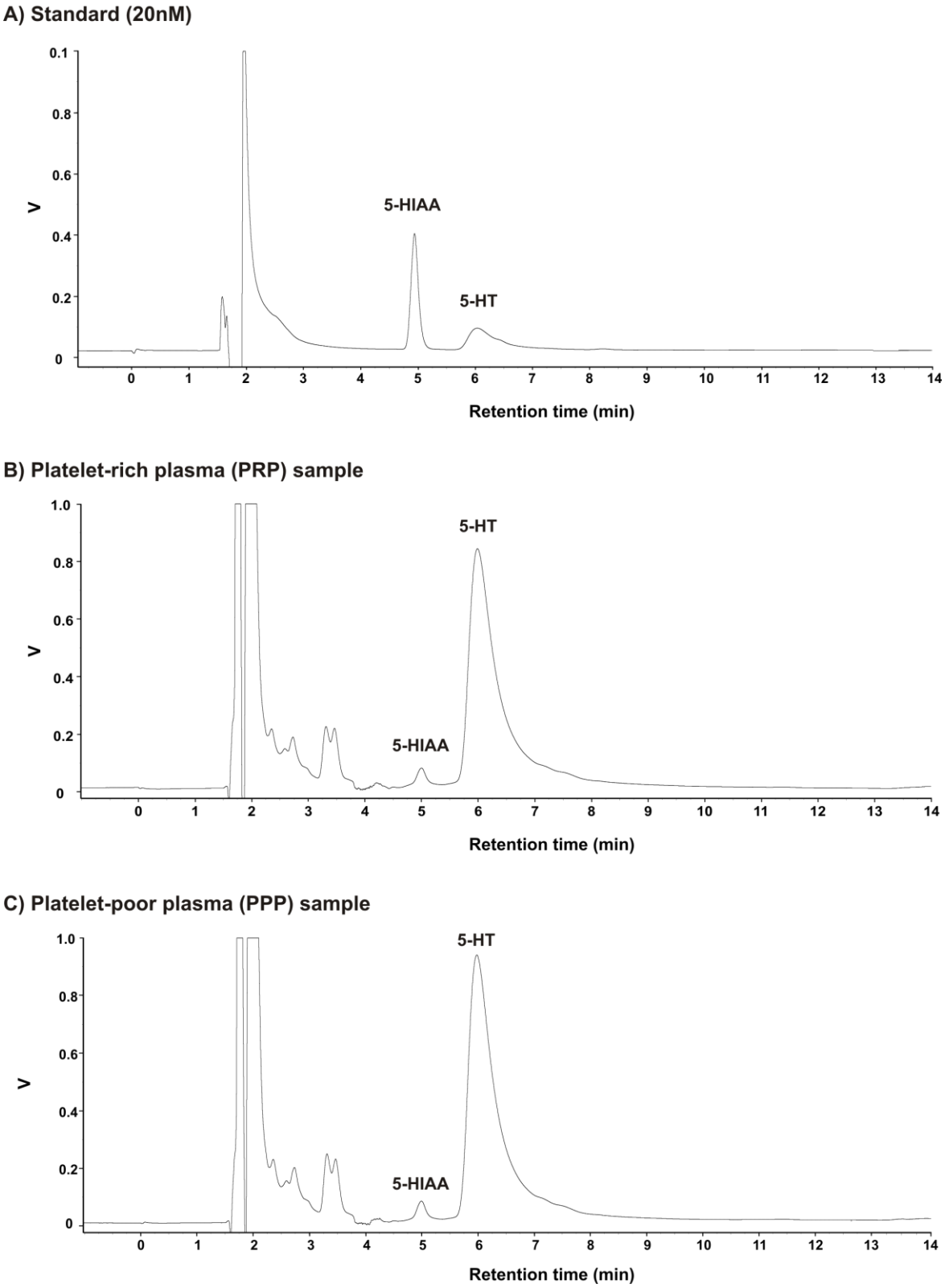

Figure 5.4: Representative HPLC chromatograms of 5-HT and 5-HIAA in rat blood plasma. A) Standard solutions for each monoamine and metabolite compound were measured via HPLC (50µl of 20nM for 5-HT and 5-HIAA) and retention times were determined. B) a platelet-rich plasma sample and C) a platelet-poor sample showing the eluted monoamine and metabolite peaks.

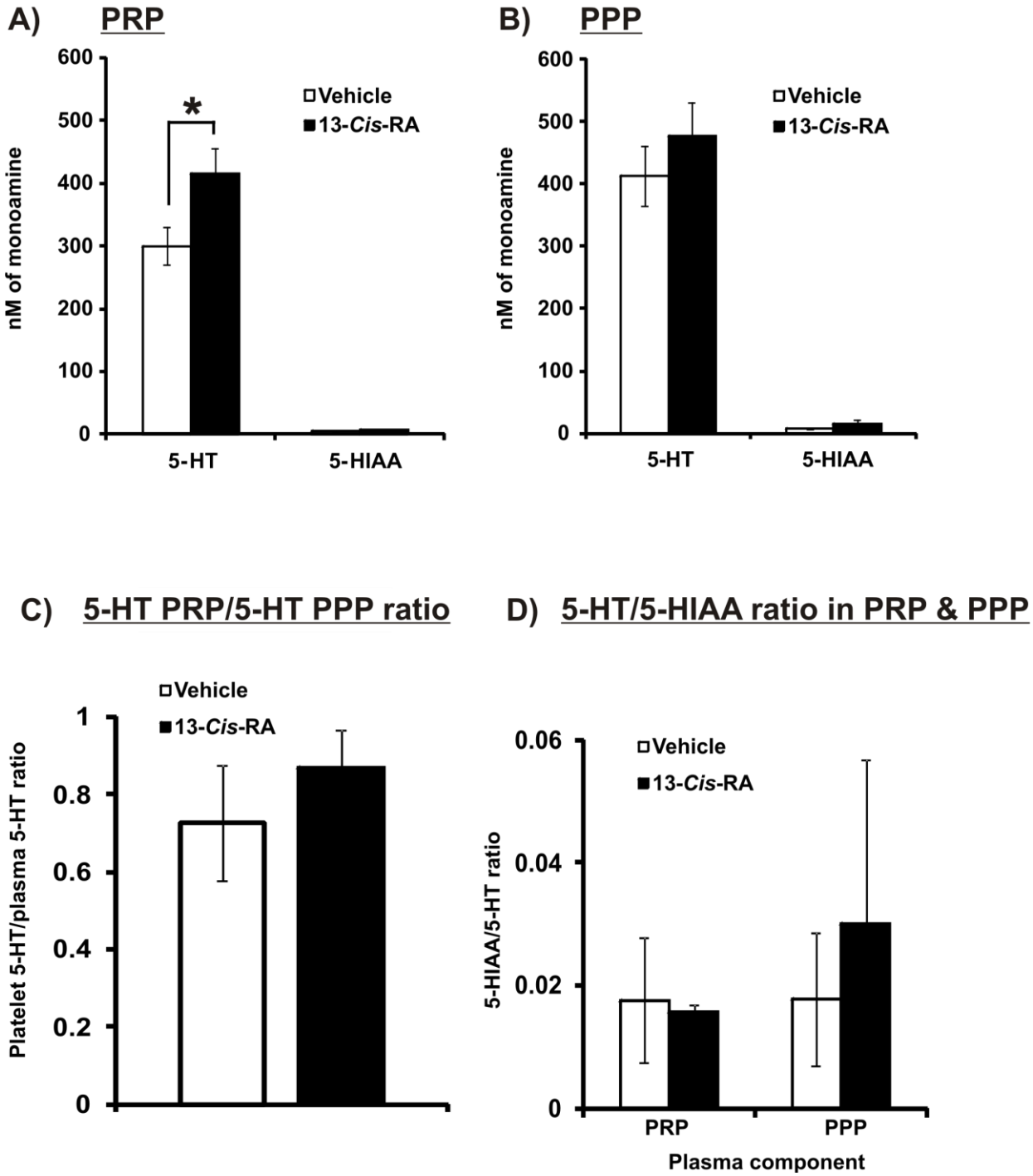

Figure 5.5: The concentration of 5-HT and 5-HIAA and turnover of 5-HT in rat PRP and PPP following chronic 13-*cis*-RA treatment. The concentration of 5-HT and 5-HIAA was measured via HPLC in A) the PRP of vehicle and 13-*cis*-RA-treated adult rats (n=12) and B) the PPP of vehicle and 13-*cis*-RA-treated adult rats (n=12). C) the ratio of platelet 5-HT/plasma 5-HT levels to give a measure of 5-HT uptake into the platelets in vehicle and 13-*cis*-RA-treated adult rats (n=12). D) 5-HIAA/5-HT was calculated in PRP and PPP to give a measure of 5-HT turnover in vehicle-treated and 13-*cis*-RA-treated adult rats (n=12). Values are mean ± SEM. * denotes $P<0.05$, t-test.

Monoamine/ metabolite	PRP		PPP	
	Vehicle	**13-*Cis*-RA**	**Vehicle**	**13-*Cis*-RA**
5-HT	300.31 ± 30.26 (12)	415.94 ± 39.39 (12)	412.65 ± 47.67 (12)	477.12 ± 53.39 (12)
5-HIAA	5.29 ± 0.59 (12)	6.59 ± 0.83 (12)	7.37 ± 0.84 (12)	14.38 ± 6.68 (12)

Table 5.2: The concentration of 5-HT and 5-HIAA in the PRP and PPP of 13-*cis*-RA-treated adult rats. PRP and PPP levels of 5-HT and 5-HIAA were determined by HPLC with electrochemical detection and values are given as nM. Values are mean ± SEM. Numbers in brackets denote n numbers of animals.

5.4. Discussion

5.4.1. 13-*Cis*-RA does not alter brain tissue monoamine levels or 5-HT turnover

The results presented in this chapter have shown that chronic 13-*cis*-RA treatment does not significantly affect the levels of 5-HT, 5-HIAA, DA or NA in the raphe nuclei, hippocampus and prefrontal cortex of adult rats. The inability of 13-*cis*-RA treatment to significantly alter brain tissue monoamine levels in these rat brain regions is in agreement the only other *in vivo* study of retinoid effects on monoamines (Ferguson *et al.*, 2005b). Ferguson *et al* found that the concentration of DA, DOPAC, HVA, 5-HT, and 5-HIAA in the prefrontal cortex and hippocampus of male and female rats treated chronically with 7.5mg/kg and 15mg/k of 13-*cis*-RA were not significantly different from vehicle-treated rats. In fact, the only significant effects of 13-*cis*-RA treatment were elevated levels of HVA and 5-HIAA in the striatum of male rats treated with 7.5mg/kg of 13-*cis*-RA compared with control males. However, the significance of alterations in HVA and 5-HIAA levels in the striatum is unclear, particularly as this brain region is not normally associated with depression pathology.

However, the striatum is highly dopaminergic and in unison with our finding that D2DR protein levels are increased in the juvenile rat hippocampus, provides putative evidence that dopaminergic systems may be affected by 13-*cis*-RA treatment. Yet, in this chapter, the DA concentration in the adult rat raphe nuclei, hippocampus and prefrontal cortex was not significantly altered by 13-*cis*-RA treatment. It is possible that the functional consequences of altered D2DR protein levels do not result in changes in DA levels in the brain regions studied. Furthermore, the significance of altered DA levels in the hippocampus in relation to depression pathology is unclear, although they may be involved in aggression. Increased levels of DA in the hippocampus may be implicated in aggression given that muricidal (aggressive mouse-killing) rats have been shown to have significantly higher DA levels in this region (Broderick *et al.*, 1985), although defensive aggression in rats has been shown to reduce DA levels in the hippocampus (Kantak *et al.*, 1984).

Our data indicates 13-*cis*-RA treatment has no effect on 5-HT levels in the raphe nuclei *in vivo*, yet this finding is in direct contrast to previous work conducted in our group utilising the RN46A-B14

raphe cell line (O'Reilly *et al.*, 2007). In this study, the raphe cell line was treated with 13-*cis*-RA (at 2.5µM and 10µM) for 48 h and 96 h, followed by HPLC analysis of intracellular 5-HT levels. The treatment of RN46A-B14 cells with 13-*cis*-RA at 10µM for 48 h led to a significant decrease in 5-HT levels, whereas 13-*cis*-RA treatment for 96 h led to a significant increase in 5-HT levels (treatment at 2.5µM had no effect after 48 h or 96 h). Therefore, the length of 13-*cis*-RA treatment *in vitro* resulted in diametrically opposing changes in 5-HT concentration, with the longer treatment resulting in increased 5-HT levels; an effect we might expect to be replicated in the chronic time course of 13-*cis*-RA administration in rats. On the surface, the *in vitro* findings of increased intracellular 5-HT concentration appear to be somewhat counterintuitive to our original hypothesis of a retinoid-induced hyposerotonergic state in the raphe nuclei. However, the same study was also able to demonstrate elevated levels of SERT protein expression, so it is therefore possible that the observed increases in 5-HT levels may simply reflect increased reuptake of 5-HT. In any case, the *in vivo* findings (no change in 5-HT concentration) do not appear to correlate with the *in vitro* findings (increases in 5-HT concentration) that suggests different neuronal pathways are affected during 13-*cis*-RA treatment and/or the cell line does not recapitulate the intricacies of the raphe nuclei and its multiple inputs and outputs to and from other neighbouring brain regions.

In this chapter we have shown no significant changes in 5-HIAA levels in the brain tissue, although there is a trend for reduced 5-HT turnover in the raphe nuclei as measured by the ratio of 5-HIAA/5-HT (P=0.081). This derives from a possible decrease in rat raphe nuclei 5-HIAA levels and may have implications for depression pathology. Human studies have recorded reductions in 5-HIAA in the CSF of depressed patients (Asberg *et al.*, 1976), although the measurement of 5-HIAA in the CSF as a potential index of depression has been inconsistent across laboratories (Cowen, 2008; Engstrom *et al.*, 1999; Reddy *et al.*, 1992) and may be more valid measure amongst suicidal depressives (Cowen, 2008). However, both Wistar and Sprague-Dawley rats undergoing the chronic mild stress paradigm were found to have reduced 5-HT turnover in the pons region that could be reversed through imipramine treatment (Vitale *et al.*, 2009). Meanhwile, numerous studies have shown a strong association between reduced 5-HIAA levels in the CSF and aggressive, impulsive and violent

behaviour in humans and nonhuman primates (Brown *et al.*, 1979; Higley *et al.*, 1992; Linnoila *et al.*, 1983; Mehlman *et al.*, 1994) and these findings have largely been confirmed in rat studies (Valzelli *et al.*, 1981; Vergnes *et al.*, 1986). The association between reduced levels of 5-HIAA and aggression potentially impacts upon our earlier finding that *13-cis*-RA treatment significantly reduces aggressive behaviour exhibited by the resident rat towards an intruder via the resident-intruder paradigm. The only brain region whereby a trend towards decreased 5-HIAA concentration was noted was the raphe nuclei and this brain region has indeed been purported to have a role in the neurobiology of aggression (van der Vegt *et al.*, 2003; Yamamoto *et al.*, 1977). However, the observed decrease in 5-HIAA concentration is unlikely to contribute to alterations in resident-rat aggression given that these rats were shown to exhibit *reduced* rather than increased levels of aggression. Meanwhile, the hippocampus is thought to have a far more prominent role in mediating aggressive behaviour (Davidson *et al.*, 2000; Gregg *et al.*, 2001; Nelson *et al.*, 2001) and we have shown the 5-HIAA concentration in this brain region remains unaffected following 13-*cis*-RA treatment. Overall, there is no significant alteration in 5-HIAA levels in the raphe nuclei that is in agreement with previous studies using the RN46A-B14 raphe cell line (2.5µM and 10µM of 13-*cis*-RA treatment for 48 h and 96 h) (O'Reilly *et al.*, 2007).

5.4.2. Changes in plasma 5-HT levels following 13-*cis*-RA administration

The results presented in this chapter have shown that chronic 13-*cis*-RA treatment significantly increased 5-HT levels in the PRP, but not in the PPP of adult rats. However, there was no concomitant change in the 5-HT platelet/5-HT plasma ratio. Meanwhile, 5-HIAA levels and 5-HT turnover were not altered in the PRP and PPP of 13-*cis*-RA-treated adult rats.

13-*Cis*-RA increased 5-HT levels in PRP of adult rats. In the periphery, the enterochromaffin cells of the gastrointestinal tract synthesise 5-HT (via TPH1) (Weber *et al.*, 1965) and excess 5-HT is stored by blood platelets through the uptake of 5-HT via SERT (Pletscher, 1987). While, platelets are unable to synthesise 5-HT themselves due to a lack of TPH (Struder *et al.*, 2001), they are able to metabolise

5-HT into 5-HIAA due to the presence of platelet mitochrondrial MAO (Pletscher, 1968), with only the MAO-B isoform present in humans (Shih *et al.*, 1999). Therefore the specific increase in 5-HT levels in the PRP, but not PPP, suggests an increase in 5-HT uptake into the platelets of 13-*cis*-RA-treated adult rats, as opposed to increased 5-HT synthesis. However, my data shows that 13-*cis*-RA treatment does not significantly increase the platelet 5-HT/plasma 5-HT ratio, suggesting increased uptake does not occur. Somewhat surprisingly, 5-HT levels do not differ between the PRP and PPP samples (in both treatment groups) and in fact, 5-HT levels are lower in the plasma and plasma (PRP) than plasma alone (PPP) in vehicle-treated rats (Figure 5.5.). This is in contrast with the general consensus that while 5-HIAA levels remain similar across PRP and PPP samples, 5-HT levels should be considerably higher in the PRP compared with the PPP of healthy humans (Ortiz *et al.*, 1988; Saracino *et al.*, 2010). This suggests that *i)* the uptake of 5-HT into the platelets is poor, *ii)* the platelets were not correctly lysed before 5-HT levels were analysed or *iii)* the PPP still contains high numbers of platelets. As a consequence, the observed increase in 5-HT levels in the PRP following 13-*cis*-RA treatment may simply derive from the underestimation of 5-HT levels in vehicles as opposed to a bona fide effect of 13-*cis*-RA treatment.

If upon further investigation, there is a significant effect of 13-*cis*-RA treatment on 5-HT levels in the periphery, the precise underlying mechanism requires elucidation. This might conceivably occur through an increase in 5-HT uptake into the platelets (that is likely to derive from an increase in SERT expression) (Mercado *et al.*, 2010; Ni *et al.*, 2006), or reduced levels of 5-HT synthesis in enterochromaffin cells of the gastrointestinal tract (perhaps via reduced TPH1 levels/activity) (Walther *et al.*, 2003a) or lastly, differences in free levels of tryptophan, the precursor to 5-HT, in the plasma (Struder *et al.*, 2001). All scenarios could lead to a hyposerotonergic state in the periphery that could reflect a hyposerotonergic in the CFS, since CSF 5-HT has been shown to be correlated with plasma 5-HT levels (Sarrias *et al.*, 1990).

Our findings of increased 5-HT levels into the platelets are in agreement with studies that have shown reduced levels of tryptophan in the plasma of depressed patients (Coppen *et al.*, 1973; Cowen *et al.*,

1989) and imply a hyposerotonergic state in the plasma. However, our results are in contrast to studies in depressed patients that have demonstrated impaired 5-HT platelet reuptake (Coppen *et al.*, 1978) and reduced [3H]-imipramine binding sites (Healy *et al.*, 1990; Wagner *et al.*, 1985). These studies suggest that 5-HT levels may, in fact, be increased in the plasma of depressed patients. Yet, the increases observed in peripheral 5-HT are not thought to indicate a similar *hyper*serotonergic state within the CNS (Meltzer, 1990) and in any case, there is no change in 5-HT levels in the PPP following 13-*cis*-RA treatment, suggesting that increased 5-HT platelet reuptake does not affect circulating 'free' 5-HT levels in the plasma.

Given that platelets are thought to resemble serotonergic neurons, it was somewhat surprising to observe a 13-*cis*-RA-mediated increase in platelet 5-HT levels but no such effect in the raphe nuclei, hippocampal and prefrontal cortex tissue of the same rats (Chapter 5.3.1.). Conversely, 13-*cis*-RA treatment did not alter 5-HIAA levels in the rat PRP, there was a trend for decreased 5-HT metabolism in the rat raphe nuclei as measured by 5-HIAA/5-HT. Increases in free tryptophan are known to increase both peripheral and central 5-HT production because the enzymes TPH and TPH2 respectively, are only half saturated (Carlsson *et al.*, 1972; Friedman *et al.*, 1972; Hamon *et al.*, 1981) and also because the 5-HT precursors tryptophan and 5-HTP are able to cross the blood brain barrier (although 5-HT itself cannot) (Chaouloff, 1989; Fernstrom *et al.*, 1972; Pardridge, 1977). It was therefore unexpected to observe such discrepancies between platelet 5-HT levels and brain tissue samples, as they suggest dissociation between peripheral and central 5-HT pathways.

5.4.3. Limitations of tissue and plasma studies

The monoamine and metabolite levels measured in the raphe nuclei, hippocampus and prefrontal cortex of vehicle-treated rats presumably reflect the normal baseline levels of the monoamines and metabolites. However, the monoamine and metabolite concentration values we have collated appear to be lower than other published studies. For instance, the prefrontal cortex levels of 5-HT and 5-HIAA in Sprague-Dawley rats were shown to be 226.9ng/g and 85.1ng/g respectively, whereas the

hippocampal levels of 5-HT and 5-HIAA were found to be 207.6ng/g and 176.0ng/g, respectively (Ferguson *et al.*, 2005b). A recent study has shown that DRN levels of 5-HT, 5-HIAA, DA and NA levels in Sprague-Dawley rats were 22µg/g, 23µg/g, 3µg/g and 20µg/g, respectively, whereas in Wistar rats monoamine levels were 15µg/g , 4µg/g , 3µg/g and 15µg/g, respectively (Scholl *et al.*, 2010). Other studies have obtained even higher concentrations such as 30mg/g, 25mg/g and 0mg/g of 5-HT, 5-HIAA and DA, respectively, in the dorsal raphe of control Albino Swiss rats (Al-Fayez *et al.*, 2005), whereas the levels of 5-HT and 5-HIAA in whole rat brain tissue have been reported to be 0.606mg/g and 0.518mg/g, respectively (Kornum *et al.*, 2006). Our values for 5-HT, 5-HIAA, DA and NA brain tissue levels were considerably lower in comparison, which suggests the degradation of monoamines during storage (for technical reasons the samples were stored for 12 months before HPC analysis). The levels of 5-HT were particularly low in all three brain regions (see Table 5.1). We would expect the concentration of 5-HT in the raphe nuclei to be considerably higher than that of the hippocampus and prefrontal cortex (see Figure 5.2D), given the high density of serotonergic neurons in the raphe nuclei, yet we did not observe this. It is therefore possible that a specific technical fault arose in the HPLC electrochemical detection of 5-HT, such that the sensitivity for 5-HT detection was too low.

We would also expect the raphe nuclei and hippocampus to only contain nominal levels of DA compared with the prefrontal cortex. Yet these trends were not observed and suggest the potential for errors in the microdissection of each specific brain region. It is conceivable that either neighbouring brain regions were mistakenly included or that parts of the correct brain region were omitted. This may be particularly relevant to the raphe nuclei brain region because of its small size, whereas the microdissected hippocampal tissue may have been contaminated with surrounding striatal tissue (it is highly dopaminergic and may account for higher than expected levels of DA).

The HPLC-led determination of monoamine alterations following 13-*cis*-RA treatment could be expanded upon in future studies. The analysis of the juvenile rat brain tissue for changes in monoamine concentration following 13-*cis*-RA treatment would have confirmed whether a) the trend

for a decrease in TPH2 protein expression found in the juvenile rat raphe nuclei would be correlated with alterations in 5-HT concentration in the juvenile rat raphe nuclei and similarly, b) the significant increase in D2DR protein levels in the juvenile hippocampus following 13-*cis*-RA treatment would correlate with altered DA levels in the juvenile rat hippocampal tissue (only the juvenile rat hippocampus revealed altered D2DR protein levels and not the adult rat hippocampus). Furthermore, an increase in n numbers may have led to trends reaching statistical significance.

5.4.4. Conclusion

In summary, 13-*cis*-RA treatment had no effect on 5-HT, 5-HIAA, NA or DA levels in the raphe nuclei, hippocampus and prefrontal of adult rats and is in agreement with the only other published *in vivo* study (Ferguson *et al.*, 2005b). This data appears to confirm that alterations in TPH2 protein levels do not occur in 13-*cis*-RA-treated animals as speculated in Chapter 4 and that increased D2DR protein levels do not affect DA levels in the brain. However, a mild trend was observed for decreased 5-HT turnover in the raphe nuclei that may reflect a hyposerotonergic state (see Chapter 6.4.). Meanwhile, 13-*cis*-RA significantly increased platelet 5-HT levels and is in agreement with our previous *in vitro* study (O'Reilly *et al.*, 2007), whereby 13-*cis*-RA treatment increased intracellular 5-HT levels of the RN46A-B14 cell-line. The study proposed that increased intracellular 5-HT levels may have been the result of increased 5-HT reuptake because SERT protein levels were elevated. It is therefore possible that 13-*cis*-RA treatment may increase SERT protein levels in platelets, thereby leading to increased 5-HT uptake into the platelets and could lead to a hyposerotonergic state in the plasma.

However, the studies of acute 5-HT via tryptophan depletion mentioned earlier, highlight one of the fundamental problems of associating monoamine levels with depression pathology. While the procedure is known to induce a relapse in depression of patients recently treated for the disorder, healthy volunteers remain unaffected (Ruhe *et al.*, 2007). The implication is that although lowered 5-HT (and NA) levels is undoubtedly a consistent feature of depression, it does not appear to be a causal

factor for depression *per se* and may instead be a downstream consequence of other primary neuronal changes. This complicates the interpretation of the data presented in this chapter and whether inferences can be made about the pro-depressive effects of 13-*cis*-RA. It may therefore be more pertinent in future studies to focus on the synaptic release of monoamines (via microdialysis techniques), which may in turn, reveal the functional effects of 13-*cis*-RA on monoaminergic neurotransmission.

Chapter 6

General Discussion

6.1 Summary of hypothesis and findings

I have investigated the controversial association between 13-*cis*-RA, an efficacious oral acne drug, and the onset of depression. To elucidate the behavioural and molecular effects of 13-*cis*-RA, I have utilised validated animal models of depression-related behaviour in parallel with molecular approaches to target underlying gene, protein and neurotransmitter changes that may be implicated with the aetiology of depression. The original hypothesis was that chronic treatment with 13-*cis*-RA would induce a pro-depressive behavioural phenotype that was associated with neuronal gene, protein and neurotransmitter changes associated with monoaminergic pathways, and in particular, serotonergic pathways. My main behavioural finding was that 13-*cis*-RA treatment reduced aggression and increased flight behaviours in adult resident rats in the resident-intruder paradigm, consistent with a pro-depressive effect. In addition, a significant increase in D2DR protein levels was evident in the juvenile hippocampus following 13-*cis*-RA treatment, although this was not accompanied by alterations in DA levels in the raphe nuclei, hippocampus and prefrontal cortex tissue. D2DR gene expression was also increased in the RN46A-B14 cell line, following 13-*cis*-RA treatment. There were also trends of increased TPH2 gene expression in the juvenile hippocampus and reduced TPH2 protein levels in the juvenile raphe nuclei, although this was not accompanied by changes in tissue levels of 5-HT or 5-HIAA in the raphe nuclei. However, 13-*cis*-RA treatment did significantly increase 5-HT levels in the platelets.

6.2 The implications of the behavioural data

I have shown that 13-*cis*-RA treatment is able to reduce resident rat aggression with a concomitant increase in flight escape and flight submit behaviour, as measured by the resident-intruder paradigm (Chapter 3). I have postulated that the behavioural phenotype observed in resident rats after 13-*cis*-RA treatment is indicative of a pro-depressive behaviour. This is based on previous resident-intruder studies that show antidepressants induce the diametrically opposite behavioural phenotype in resident rats compared with those treated with 13-*cis*-RA (ie antidepressants increase resident rat aggression with a concomitant reduction in flight behaviour) (Mitchell *et al.*, 2005). Antidepressant-induced

increases in resident rat aggression are thought to model depressed humans undergoing antidepressant treatment that exhibit a shift from inwardly directed aggressive behaviour (guilt, remorse, suicidal ideation, suicidal acts and completed suicide) to outwardly directed aggressive behaviour (nonverbal and verbal communication, assertiveness and increased sociability) (Mitchell, 2005). Therefore, the reduction in resident rat aggressive behaviour following 13-*cis*-RA treatment is likely to reflect the converse shift, from outwardly directed aggressive behaviour (extrapunitive aggression) to inwardly directed aggressive behaviour (intropunitive aggression) in 13-*cis*-RA-treated human patients and could conceivably lead to depressive symptoms. Whether it is justifiable to suppose that the diametrically opposite behavioural phenotype in the resident-intruder following antidepressant treatment paradigm relates to a prodepressive effect of treatment is not clear without further evidence. However, similar inferences have been made in the FST whereby procedures that increase immobility times have been regarded as pro-depressive, based solely on the fact that antidepressants are known to reduce immobility (Tasset *et al.*, 2008; Zaniewska *et al.*, 2010).

Furthermore, I have found that 13-*cis*-RA treatment increased resident-rat flight behaviour; a behaviour that has consistently been observed in the ethological studies of depressed patients, whereby increased flight behaviour (and impaired sociability) were observed in the form of fewer facial expressions and gestures that instigate or invite social interactions, thereby leading to social isolation (Dixon *et al.*, 1989). Meanwhile, ethological studies have demonstrated that the abnormal behavioural responses of depressed patients to environmental and social stimulation are progressively modified during remission from the illness (Eisen, 1989; Khan *et al.*, 1989; Oswald *et al.*, 1972), with increasingly reduced self-criticism and feelings of guilt (Priest *et al.*, 1980) that leads to increased physical and/or verbal interaction with environmental and social events (Kaplan *et al.*, 1961). Therefore, the reduced flight behaviour in the resident-intruder paradigm following chronic antidepressant treatment accurately models this reversal of impaired sociability and forms an important feature of the recovery process from depressive illness.

The observation that aggression levels could be altered by 13-*cis*-RA treatment in resident rats suggests the involvement of 5-HT pathways (Nelson *et al.*, 2001; Popova, 2006). Generally, low 5-HT levels are associated with higher levels of impulsivity and aggressiveness (Birger *et al.*, 2003; Coccaro *et al.*, 1997), while manipulations that lower 5-HT signalling such as the neuronal nitric oxide synthase knockout mouse (with reduced 5-HT turnover and impaired 5-HT_{1A}R and 5-HT_{1B}R function) increase impulsivity and aggression (Chiavegatto *et al.*, 2001). Conversely, increasing 5-HT levels with 5-HT precursors, SSRIs, 5-HT_{1A}R agonist or 5-HT_{1B}R agonists has been shown to reduce aggressive behaviour in rodents (Miczek *et al.*, 2001). Therefore, serotonergic pathways are likely to be involved in the altered aggression levels of 13-*cis*-RA-treated rats. However, 13-*cis*-RA treatment reduced resident-rat aggression behaviour indicating increased 5-HT levels that is contrary to the proposed hyposerotonergic state associated with depression.

It is therefore conceivable that human patients undergoing 13-*cis*-RA treatments may similarly be susceptible to developing depression and may display changes in aggression; both of which would derive from altered serotonergic mechanisms. However, such a conclusion needs further clarification at the clinical level. Firstly, SSRIs are used in the treatment of human impulsive aggression (Coccaro *et al.*, 1997; Evenden, 1999) and is therefore in direct contrast with the resident-intruder paradigm that shows increased aggression following chronic antidepressant treatment. This paradox is partly explained by the ability of antidepressants to both increase aggression in submissive depressed individuals, whereby intropunitive aggression and/or impaired sociability are reversed (Dixon *et al.*, 1989; Priest *et al.*, 1980), and decrease pathological aggression (Hollander, 1999). It is therefore thought that antidepressant treatment increases assertiveness in human patients as this would both increase low levels of social dominance and also decrease high levels of physical aggression (Mitchell, 2005; Mitchell *et al.*, 2005). In turn, the ability of chronic antidepressant treatment to increase assertiveness and replace intropunitive aggression with extrapunitive aggression during the recovery of depression is reflected by the increased levels of aggression in rats in the resident-intruder paradigm.

Secondly, there is no evidence from patient data that 13-*cis*-RA treatment alters aggression, although this may be due to the incompleteness of patient data mentioned in Chapter 1.3.2. or the subtly of such changes in aggression/assertiveness. A future study whereby the aggression and assertiveness levels of 13-*cis*-RA patients were assessed would be of great benefit. This would likely involve the close monitoring of pre-treatment, during treatment and post-treatment patient behaviour using a self-assessment form such as the Rathus Assertiveness Scale (Burkhart *et al.*, 1979) or diagnosis from the clinician. This may provide convincing evidence of the association between retinoids and altered levels of assertiveness in humans that would closely follow our resident-intruder data and would further suggest the onset of pro-depressive behaviour in 13-*cis*-RA human patients.

The behavioural studies I have conducted in this thesis were also designed to understand the discrepancies that existed between our previous work (O'Reilly *et al.*, 2006) and that of others (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b). The apparent lack of behavioural effect in adult rats (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b) suggested that the behavioural effects of 13-*cis*-RA may be age specific (adult vs juvenile), species specific (rats vs mice), dose specific (7.5 or 30mg/kg/day vs 1mg/kg/day) or specific to the route of administration used (oral gavage vs intraperitoneal injection). I have shown that 13-*cis*-RA had no effect on the FST and sucrose consumption test in adult rats (1mg/kg/day, i.p.); a finding that was identical to the study by Ferguson despite the differences in doses and route of administration used (Ferguson *et al.*, 2005a; Ferguson *et al.*, 2007b). It therefore appears unlikely that the depressive effects previously observed by O'Reilly in juvenile mice in the FST and TST (O'Reilly *et al.*, 2006) relate to differences in doses and route of administration. Furthermore, we have shown that 13-*cis*-RA produces a depressive-like behavioural profile in adult rats in the resident-intruder paradigm suggesting that 13-*cis*-RA can induce depressive phenotypes in both mice and rats. It is therefore possible that the different behavioural effects of 13-*cis*-RA treatment seen in the two studies derive from an age-specific effect.

The importance of determining whether age is a risk factor for 13-*cis*-RA-induced depression is underscored by the fact that older juveniles (15-19 years) represent the largest demographic group of

13-*cis*-RA patients (Wysowski *et al.*, 2002) and emerging data that some of the neurobiological correlates of juvenile depression differ from those involved in adult depression (Kaufman *et al.*, 2001). However, there is a lack of studies that have compared the incident rates of depression, suicide ideation and completed suicide in adult and juvenile 13-*cis*-RA patient groups (although both age groups are known to be affected (Byrne *et al.*, 1998; Duke *et al.*, 1993; Strahan *et al.*, 2006)). Meanwhile, I have employed the FST and sucrose consumption test to examine behaviours in juvenile and adult retinoid-treated animals and found no effect of 13-*cis*-RA treatment in either tests, while it was not possible to conduct the resident-intruder paradigm in juveniles. As a consequence, it has not been possible to exclude age as a factor.

Moreover, all the behavioural tests employed have been validated in adult animals as opposed to juveniles. For instance, the ability of antidepressant treatment to reduce immobility in the FST in juvenile animals is controversial (Reed *et al.*, 2008) and furthermore, fluoxetine is the only antidepressant that has been shown to be efficacious in the treatment of juvenile depressive patients (Emslie *et al.*, 2005b; Kutcher *et al.*, 1994; Kye *et al.*, 1996). This is best demonstrated by a recent study that was able to show that administration of TCAs (desipramine and imipramine) to 21-day-old rats did not decrease immobility and therefore did not have antidepressive-activity, whereas SSRI treatment (fluoxetine and escitalopram) was effective at reducing immobility (Reed *et al.*, 2008). Paradoxically, the TCA desmethylimipramine has been shown to reduce immobility in 30 day old rats despite the fact it has no clinical efficacy in human juvenile depression (Pechnick *et al.*, 2008). Therefore, the age-specific effects of 13-*cis*-RA remain cannot be discounted because a) the comparison between 13-*cis*-RA-treated juvenile mice against adult mice in the FST have not been conducted and b) the FST may not be a suitable model for measuring the age specific effects of 13-*cis*-RA and another test is therefore required. Given that a species-specific effect is unlikely, as 13-*cis*-RA is capable of inducing pro-depressive effects in mice and rats (albeit in different behavioural tests), I believe the discrepancies in results obtained from us and others derive from an age specific effect of 13-*cis*-RA that may partially depend upon the particular depression-related behavioural test used. This is also further substantiated by the finding that the only significant molecular alterations

caused by 13-*cis*-RA occurred in juvenile rats (altered D2DR protein levels, trends for altered TPH2 gene and protein levels).

6.3 The monoaminergic components associated with altered resident-intruder behaviour

The finding that 13-*cis*-RA treatment alters resident rat behaviour, as measured by the resident-intruder paradigm, provides indirect evidence for the involvement of the 5-HT_{1A}R and the 5-HT_{2C}R. The ability of fluoxetine to induce increased aggression/reduced flight behaviour in resident rat behaviour was found to be potentiated by co-administration of 5-HT_{1A}R antagonist WAY-100635 (altered behaviour was observed after 2 days with fluoxetine and the antagonist vs 5 days with just chronic fluoxetine treatment) (Mitchell *et al.*, 1997b). This finding implicates the 5-HT_{1A}R with the onset of the increase in aggression behaviour and moreover, suggests that reduced resident rat aggression may derive from increased 5-HT_{1A}R function. Importantly, this is identical to the previous *in vitro* work conducted in our group, whereby 13-*cis*-RA treatment was shown to upregulate 5-HT_{1A}R protein levels (O'Reilly *et al.*, 2007). Therefore, the 5-HT_{1A}R may represent the common molecular component that mediates the behavioural change observed in the resident-intruder paradigm and increased susceptibility to depression exhibited by 13-*cis*-RA patients. However, the data from rats undergoing 8-OH-DPAT-induced hypothermia (Chapter 3) revealed that 13-*cis*-RA treatment had no functional changes in 5-HT_{1A}R and protein levels were not altered *in vivo* (Chapter 4). Therefore, my data does not support the involvement of 5-HT_{1A}R in 13-*cis*-RA-induced depression, despite the finding that resident-rat behaviour is altered by 13-*cis*-RA.

Meanwhile, previous resident-intruder studies have found temporal associations between the antidepressant-induced increases in aggression of resident rats and reductions in 5-HT_{2C}R-mediated function measured by hypolocomotion induced by *m*CPP (Mitchell *et al.*, 2003; Mitchell *et al.*, 2000a). Furthermore, the acute administration of mesulergine, a 5-HT_{2C}R antagonist, induced an increase in aggressive resident rat behaviour that was qualitatively similar to that seen with chronic

antidepressants with *m*CPP (Mitchell *et al.*, 2000b). These findings are highly relevant given that the 5-HT_{2C}R has implications in antidepressant action (Berg *et al.*, 2008; Di Giovanni *et al.*, 2006; Serretti *et al.*, 2004), as well as aggression in other behavioural models. The novel 5-HT_{2C}R agonist, WAY-163909, was found to reduce immobility times in the FST (Rosenzweig-Lipson *et al.*, 2007), although the 5-HT_{2C}R antagonists S32006 and agomelatine have similarly been shown to have antidepressive effects (Dekeyne *et al.*, 2008; Popoli, 2009). Meanwhile, a form of aggressive behaviour in cats known as defensive rage, is thought to be mediated by 5-HT_{2C}Rs in the midbrain periaqueductal gray (Bhatt *et al.*, 2008), while the selective 5-HT_{2C}R antagonist S32006 inhibits aggressive behaviour in mice (Dekeyne *et al.*, 2008). Furthermore, rats bred for an absence of high levels of stress-evoked aggression to humans had higher levels of 5-HT_{2C}R mRNA in the frontal cortex and hippocampus compared with rats bred for the presence of high levels of stress-evoked aggression to humans (Popova *et al.*, 2010). However, knockout studies appear to demonstrate that 5-$HT_{2C}R^{-/-}$ mice do not exhibit altered aggression levels, unlike $MAOA^{-/-}$ and 5-$HT_{1B}R^{-/-}$ mice which display increased aggression (Heath *et al.*, 1995). Overall, resident-intruder studies have previously highlighted the association between the 5-HT_{2C}R and aggressive behaviour, whereas other studies suggest the involvement of 5-HT_{2C}R in antidepressant action. Together, this strengthens the possibility that the alteration in behaviour following 13-*cis*-RA treatment is of a depressive nature and reflects a change towards increased 5-HT_{2C}R-mediated function. It is not currently known whether such an association exists, although this could be further established by future molecular studies to investigate 5-HT_{2C}R expression changes in the brain tissue of 13-*cis*-RA-treated rats.

The most thorough attempts to characterise the molecular alterations that underlie changes in resident-rat behaviour have focused on the 5-HT_{1A}R, 5-HT_{2A}R and 5-HT_{2C}Rs, and as a consequence, have suggested altered serotonergic pathways may be involved (Mitchell, 2005). However, it is worth noting the ability of certain drugs, with dopaminergic pharmacological actions, to alter resident-rat behaviour. For instance, the chronic treatment of resident rats with amphetamine, a drug thought to act by inhibiting the dopamine transporter (Kahlig *et al.*, 2003), can increase flight behaviour (although aggressive behaviour remained unchanged) (Mitchell *et al.*, 1997a). Meanwhile, the chronic

treatment of resident rats with haloperidol, a D2DR antagonist (Boulay *et al.*, 2000; Bunzow *et al.*, 1988), resulted in decreased aggression and increased flight behaviour (Mitchell *et al.*, 1992a). It is therefore possible that the ability of 13-*cis*-RA to alter resident-rat behaviour might indicate alterations in the dopaminergic pathways, as opposed to the serotonergic pathways.

6.4. The implications of potentially reduced TPH2 levels in 13-*cis*-RA-treated rats

The *in vivo* studies revealed trends for a reduction in protein levels of TPH2 in the rat raphe nuclei following 13-*cis*-RA treatment (Chapter 4). However, the study of brain tissue levels of monoamines revealed that there were no corresponding reductions in 5-HT and 5-HIAA in the raphe nuclei, although the turnover of 5-HT did appear to be reduced in this region (Chapter 5). There is evidence to suggest that reduced TPH2 expression and/or activity can lead to depression-related behaviour in animals. For instance, the reduced TPH2 expression displayed in TPH2 knockout mice (Savelieva *et al.*, 2008) and reduced activity of TPH2 in TPH2 R441H knockin mice (Beaulieu *et al.*, 2008) both resulted in increased immobility times in the tail suspension test. However, it should also be noted that reductions in TPH2 have been associated with the opposite trend: a reduction in depression-related behaviour in animals. For example, the C1473G polymorphism of the TPH2 gene in mice reduces TPH2 enzyme activity, reduces aggression levels and reduces immobility times in the FST of mice (Osipova *et al.*, 2009). Another study shows that male, but not female, TPH2 knockout mice display reduced immobility in the FST (Savelieva *et al.*, 2008). So reductions in TPH2 expression may not be conclusively associated with prodepressive effects in animals *per se*, although the involvement of TPH2 would appear to be quite convincing.

Similarly contradictory findings occur in the literature between TPH2 levels and depression in humans. Human post-mortem studies have shown that depressed suicides have a 33% increase in TPH2 mRNA expression in the dorsal raphe nuclei (Bach-Mizrachi *et al.*, 2006) and depressed suicides have a greater density and number of TPH-immunoreactive neurons in the dorsal raphe nuclei (Bach-Mizrachi *et al.*, 2008; Underwood *et al.*, 1999). However, these alterations are not

congruent with our findings or with the general consensus that depression arises through a hyposerotonergic state. The authors argued that this apparently paradoxical increase may be a homeostatic response to reduced levels of 5-HT thought to occur in depressed patients or it may be that the isoenzyme form of TPH2 has reduced activity and therefore does not result in increased 5-HT synthesis. In contrast, a human polymorphism of the TPH2 promoter known as rs11178997, that has been linked to major depression (Zhou *et al.*, 2005), has been shown to reduce the TPH2 transcriptional activity in primary raphe neurons (Scheuch *et al.*, 2007). In summary, the field associating TPH2 with depression is confused and contradictory in both human and animal studies.

As an aside, the study of TPH2 mRNA expression in human suicide victims (Bach-Mizrachi *et al.*, 2006), highlights the understandable bias that exists towards the study of depressed completed suicide victims, as opposed to depressed patients, when requiring human brain tissue. Although completed suicide is generally regarded as a serious progression from suicide ideation, which is itself a serious progression from clinical depression, it is unlikely that they are characterised by identical monoaminergic/neuronal pathways and could be quite mechanistically diverse. It is therefore still unknown whether non-suicidal depressed patients exhibit reductions in TPH2 protein, as suggested by our animal studies, or increases as suggested by mRNA data from human suicide victims (Bach-Mizrachi *et al.*, 2006).

TPH2 is intrinsically associated with 5-HT synthesis, demonstrated by the 96% reduction in 5-HT in the dorsal raphe of TPH2 knockout mice (Alenina *et al.*, 2009) and similarly, the 95% reduction in 5-HT in the brain stem of TPH2 knockout mice (Savelieva *et al.*, 2008). It is therefore reasonable to suppose that a similar correlation between TPH2 and 5-HT levels exists in the human brain. However, there are presumably a number of factors that must remain constant for such a correlation to exist. Firstly, the unsaturated state of the TPH2 enzyme under physiological conditions (Hamon *et al.*, 1981) (Carlsson *et al.*, 1972) means that alterations in the levels of substrate, tryptophan, could have significant consequences on 5-HT synthesis. Additionally, alterations in the activity (Vmax) of the

TPH2 enzyme may occur, such that the positive correlation between TPH2 expression levels and the rate of 5-HT synthesis is altered.

Furthermore, there are two potentially large confounds with the interpretation of the molecular and neurotransmitter data. Firstly, the significant TPH2 alterations we observed in the raphe nuclei derived from rats who displayed no behavioural alterations in response to 6 weeks of 13-*cis*-RA treatment as measured by the FST and sucrose consumption test. Meanwhile, we observed that two weeks of 13-*cis*-RA treatment was sufficient to induce depression-related behaviour in the resident-intruder paradigm. However, no molecular or neurotransmitter analyses were conducted in these resident rats due to the cessation of 13-*cis*-RA treatment for one week (post-treatment, to assess the behavioural effects of de-challenge). Therefore, the effects of two and six weeks 13-*cis*-RA treatment on the molecular/neurotransmitter and behavioural changes of resident-rats in the resident-intruder paradigm have not been tested. Similarly, no molecular or neurotransmitter analyses were conducted in rats undergoing the FST and sucrose consumption test after two weeks of 13-*cis*-RA treatment. We therefore have a divergence in the data between when we observe the pro-depressive effects of 13-*cis*-RA (2 wk) and when we observe molecular alterations in 13-*cis*-RA-treated animals (6 wk). A time-course study of the molecular changes and neurotransmitter levels would provide evidence of a) whether monoaminergic changes occur at two weeks of 13-*cis*-RA treatment, in line with the resident-intruder behavioural data and b) detailed information of the molecular/neurotransmitter changes that may occur in the intervening weeks.

A second potential confound of our molecular and neurotransmitter data is the inability to discriminate between the different subregions of the raphe nuclei: the dorsal and median raphe nuclei. Although both regions contain the majority of the serotonergic neurons that innervate forebrain regions (Azmitia *et al.*, 1978), they are quite distinct from each other (Lechin *et al.*, 2006). The dorsal raphe is mainly serotonergic, thought to contain in the region of 10,000-12,000 5-HT neurons (Descarries *et al.*, 1982), and innervates the fronto-parietal cortex, ventral hippocampus, amygdala, lateral septum, nuclei accumbens shell, substantia nigra, striatum and hypothalamus (Hornung, 2003;

Lechin *et al.*, 2006). Meanwhile, in the median raphe nuclei, only a small proportion of neurons are serotonergic (Wiklund *et al.*, 1981), and innervate the temporal cortex, dorsal hippocampus, central amygdala, medial septum, nuclei accumbens core, ventral tegmental area, mesolimbic structures and hypothalamus (Hornung, 2003; Lechin *et al.*, 2006). Furthermore, the findings that TPH2 expression are increased in depressed suicides appears to be restricted to the dorsal raphe nuclei (Boldrini *et al.*, 2005; Underwood *et al.*, 1999), whereas the increase of TPH2 in the median raphe nuclei is less consistent (Bach-Mizrachi *et al.*, 2006). Therefore, the microdissection of the entire rat raphe nuclei tissue containing both the dorsal and median raphe nucleus may have masked local regional differences within the raphe nuclei that could potentially confound the interpretation of the gene, protein and neurotransmitter concentration data.

In future studies, the mechanism through which 13-*cis*-RA treatment might be able to reduce TPH2 protein expression will require elucidation. Generally, retinoids have been shown to elevate neuronal gene transcription through the binding of RAR/RXR heterodimers to RAREs (Lane *et al.*, 2005) and indeed, the evidence from this thesis suggests a trend for elevated TPH2 gene expression by 13-*cis*-RA treatment. However, the distinct dissociation between reduced TPH2 protein levels and increased gene transcription suggests other post-transcriptional mechanisms or post-translational modifications may occur. Alternatively, reduced TPH2 protein levels may be reflected by reduced TPH2 gene expression and were simply not accurately observed in this thesis due to methodological error. In this instance, 13-*cis*-RA may bind to other receptors that compete with the RAR/RXR heterodimer to bind to the RARE, thereby reducing gene transcription, or 13-*cis*-RA may regulate transcription factors that are able to regulate TPH2 expression. In the former scenario, such receptors might include the COUF-TF receptor or the nuclear orphan receptors TOR and Tak-1 which inhibit retinoid-induced gene transcription (Hirose *et al.*, 1995; Ortiz *et al.*, 1995; Tran *et al.*, 1992), although it is not known whether 13-*cis*-RA binds to receptors other than RARs. At present, the sequencing of TPH2 gene has not revealed a RARE in the promoter that would have inferred the ability of retinoids to regulate TPH2 gene transcription (Walther *et al.*, 2003b) and the same applies to all GOI studied in this thesis, with the exception of D2DR and 5-HT_{1A}R (see Chapter 4.1.1.).

Like retinoic acid receptors, glucocorticoids are members of the nuclear receptor superfamily and initiate gene transcription through a glucocorticoid response element (Kumar *et al.*, 2005). Intriguingly, glucocorticoids have been implicated with the regulation of TPH2 mRNA expression (Clark *et al.*, 2005) and TPH2 protein levels (Clark *et al.*, 2008) in the mouse raphe nuclei. In both studies, the glucocorticoid receptor agonist dexamethasone has been shown to reduce both TPH2 mRNA and protein levels in the DRN and the effect could be reversed by the co-administration of the glucocorticoid receptor antagonist mifepristone (Clark *et al.*, 2008; Clark *et al.*, 2005). Moreover, the glucocorticoid-mediated reduction in TPH2 protein levels was found to have functional consequences in 5-HT biosynthesis, given that significant reductions in 5-HTP were observed in the frontal cortex (Clark *et al.*, 2008). The ability of 13-*cis*-RA to alter TPH2 expression may therefore occur in a similar fashion or may derive from cross-talk between retinoid and glucocorticoid signalling systems, perhaps by the formation of a heterodimer between the RXR and the glucocorticoid receptor.

Ultimately, the trend for a reduction in TPH2 protein levels seen in the raphe nuclei of 13-*cis*-RA-treated rats was not reflected in lowered 5-HT and 5-HIAA levels in the raphe nuclei, although there was a trend for the turnover of 5-HT to be reduced in this region. The possible finding that intracellular 5-HT turnover was reduced indicates reduced levels of 5-HT were metabolised to 5-HIAA in the neurons of the raphe nuclei. However, it is not clear if the reduced intracellular turnover of 5-HT is similarly reflected by a reduction in synaptic turnover of 5-HT, which might indicate reductions in 5-HT release and postsynaptic activation. Alternatively, reduced levels of intracellular 5-HT turnover in the raphe nuclei might be indicative of a negative feedback system following an *increase* in 5-HT synaptic activity. Until microdialysis studies are conducted, understanding the effects of 13-*cis*-RA treatment on 5-HT synaptic release, and therefore activity, will not be possible.

6.5 Other molecular mechanisms that may underlie retinoid-induced depression

This thesis provides weak and indirect evidence of serotonergic components (TPH2, 5-HT_{1A}R and 5-HT_{2C}R) as the potential molecular targets underlying 13-*cis*-RA-induced depression. However, other monoaminergic pathways (DA and NA) may provide promising avenues of research, given the data presented and considerable body of literature implicating DA/NA with depression (Brunello *et al.*, 2002; Nestler *et al.*, 2006). Dopaminergic pathways are implicated given that my work shows D2DR gene and protein levels are significantly elevated, whereas the highly dopaminergic striatum (Dailly *et al.*, 2004) has been shown by others to be the site of elevated levels of HVA and 5-HIAA following 13-*cis*-RA treatment (Ferguson *et al.*, 2005b). Furthermore, my finding that 13-*cis*-RA alters resident-rat aggression implicates both dopaminergic and noradrenergic systems because the most enduring pharmacological intervention of human aggression involves DA receptor antagonists (reviewed in (de Almeida *et al.*, 2005)) and positive correlations between CSF levels of NA and aggressiveness have been observed in humans (Brown *et al.*, 1982; Placidi *et al.*, 2001). Yet, HPLC analysis revealed no changes in DA levels in the raphe nuclei, hippocampus or prefrontal cortex that is not in agreement with our gene expression and protein level findings. The involvement of noradrenergic pathways was not thoroughly assessed in this thesis (adrenergic receptor expression/function was not studied) and therefore its involvement cannot be fully discounted, although the HPLC analysis of NA levels in the raphe nuclei, hippocampus and prefrontal cortex tissue highlighted the inability of 13-*cis*-RA to affect this monoaminergic system.

Clearly, more in depth studies focusing on dopaminergic and noradrenergic pathways are required, and it is likely that a complex interaction of pathways and molecular are responsible for the 13-*cis*-RA-induced onset of a highly complex and heterogeneous behaviour such as depression. For instance, it may be possible that it is in fact the serotonergic regulation of dopaminergic pathways (Alex *et al.*, 2007) and noradrenergic pathways (Fink *et al.*, 2007) in the brain that is of importance.

Currently, the best explored non-monoaminergic mechanism that may underlie 13-*cis*-RA-induced depression is the inhibition of neurogenesis (Crandall *et al.*, 2004); a process implicated with depression pathology and antidepressant action (described in Chapter 1.3.1.6.). Mice treated daily for 6 weeks with 1mg/kg of 13-*cis*-RA were found to have significant reductions in cell proliferation within the hippocampal and subventricular zone, significant reductions in cell survival within the hippocampal formation and subgranular zone and a decline in neurogenesis (Crandall *et al.*, 2004). How 13-*cis*-RA might influence neurogenesis is not currently known. One possibility is that 13-*cis*-RA reduces the levels of BDNF which is essential for neurogenesis (Ghosh *et al.*, 1994; Lindholm *et al.*, 1996). Moreover, serum levels of BDNF have been shown to be reduced in depressed patients (Karege *et al.*, 2002), while increased post-mortem BDNF expression was found in dentate gyrus and supragranular regions in patients treated with antidepressants compared with non-untreated patients (Chen *et al.*, 2001). Other mechanisms through which 13-*cis*-RA might reduce neurogenesis include changes in the expression of the neurotrophic factor receptor TrkB (Edsjo *et al.*, 2003; Pombo *et al.*, 2000) or the apoptotic nature of retinoids and therefore 13-*cis*-RA (Crandall *et al.*, 2004; Guillemain *et al.*, 2003; Ninomiya *et al.*, 1997). Work currently ongoing within our own group may uncover how 13-*cis*-RA is able to inhibit murine neurogenesis and in the process, would further strengthen the hypothesis that 13-*cis*-RA can cause the onset of depression.

The hypothalamic-pituitary-adrenal axis has been implicated in depression pathology (see Chapter 1.3.1.6.) and there is some evidence that the HPA axis is also sensitive to retinoids. Firstly, several hypothalamic proteins are thought to be regulated by retinoic acid (Breen *et al.*, 1997; Cho *et al.*, 2001; Richard *et al.*, 1991) and components of the retinoid signalling cascade such as CRBP-I, CRABP-I, RARγ and RXRγ are present in the hypothalamus (Krezel *et al.*, 1999; Zetterstrom *et al.*, 1999). Furthermore, the expression of the retinoid receptor RARα has been shown to colocalise with corticotrophin-releasing hormone (CRH) expression in the neurons of the human hypothalamic paraventricular nuclei and the immunoreactive densities of RARα-CRH double labelled neurons were elevated in patients with affective disorders compared to controls (Chen *et al.*, 2009). The same study was also able to show that RARα is recruited by the CRH promoter in the rat hypothalamus and in the

human neuroblastoma cell line BE(2)-C, while RARα was also able to elevate CRH mRNA expression in BE(2)-C cells (Chen *et al.*, 2009). Meanwhile, another study has found that retinoic acid treatment of hypothalamic slice cultures (from postnatal day 7 rats) elevates the number of cells labelled with the neuroendocrine peptide adrenocorticotropin hormone, although no such changes were seen in CRH expression (Shearer *et al.*, 2010). It therefore appears as though retinoids are able to influence components of the HPA and could represent the underlying mechanism of retinoid-induced depression, although the effects of 13-*cis*-RA itself are not presently known.

6.6 Final conclusion

I believe this thesis presents evidence of the ability of 13-*cis*-RA to induce behavioural effects in rats and modest evidence of molecular alterations in rats. The behavioural changes are consistent with a pro-depressive effect of 13-*cis*-RA which reflects the postulated retinoid-induced depression thought to occur in humans. The initial hypothesis that 13-*cis*-RA treatment can lead to pro-depressive behaviour through alterations in the monoaminergic pathways remains viable, although the particular role of serotonergic pathways is questionable given the data presented in this thesis. While behavioural changes in aggression in the resident-intruder paradigm suggest increased serotonergic activation following 13-*cis*-RA treatment (although dopaminergic pathways may also be involved), the only observed serotonergic molecular alterations were a significant increase in 5-HT levels in platelets and trends for altered TPH2 levels (findings summarized in Figure 6.1). The increased level of 5-HT in platelets does add to previous work in our group whereby increased 5-HT reuptake is thought to occur following 13-*cis*-RA treatment *in vitro*, whereas the putative evidence of reduced 5-HT synthesis (in the form of lowered TPH2 protein levels) and reduced 5-HT turnover is based on weak trends in my data and would require further investigation. Indeed, it could be argued that 13-*cis*-RA-induced depression does not involve serotonergic signalling components given the inability for SERT and 5-HT_{1A}R protein levels to be altered, 5-HT_{1A}R function to be altered (via 8-OH-DPAT-induced hypothermia studies), 5-HT_{1B}R gene expression to be significantly altered and TPH2 protein levels to be altered significantly.

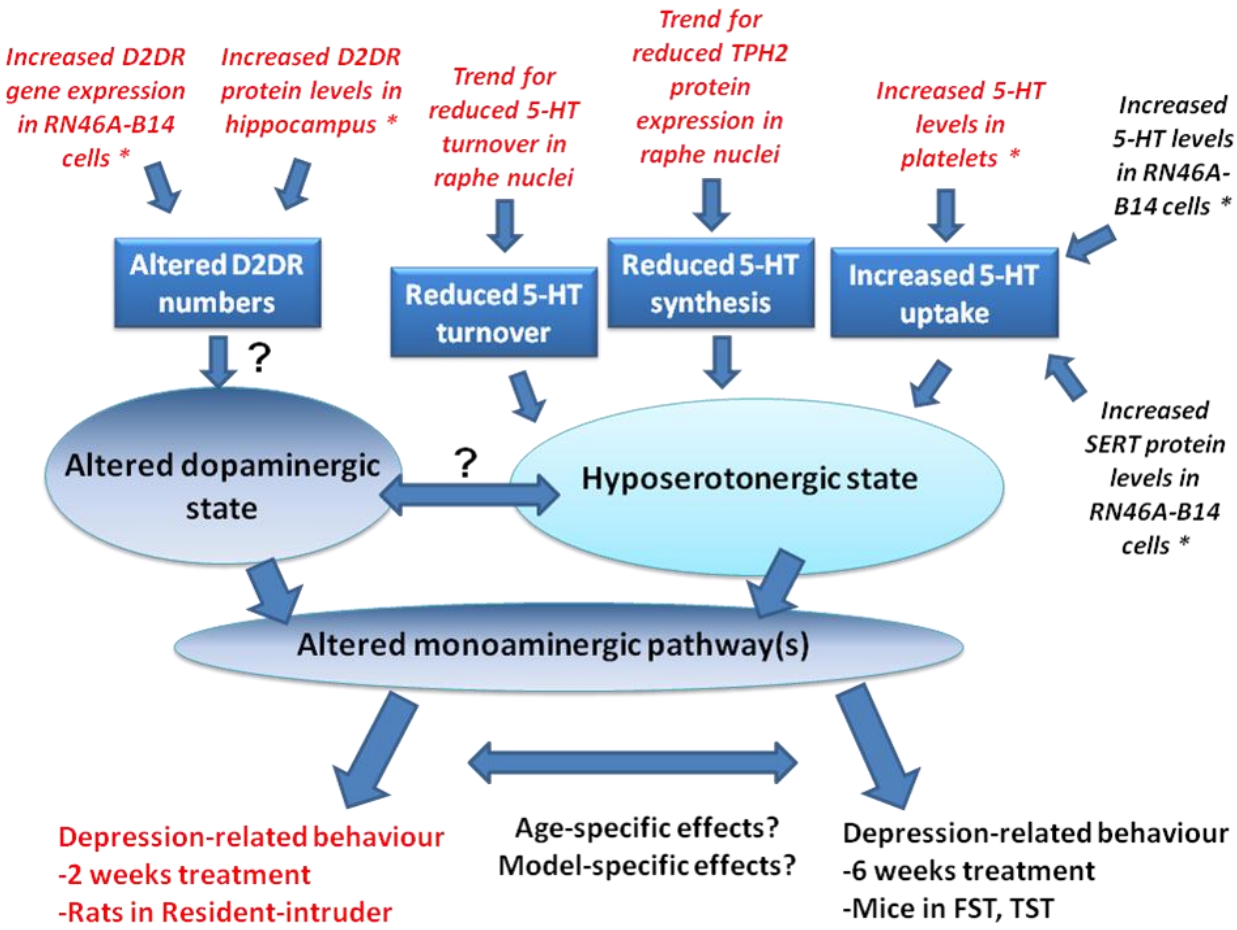

Figure 6.1: Current understanding of the molecular mechanisms underlying 13-*cis*-RA-mediated depression. A hyposerotonergic state or alterations in the dopaminergic pathway are hypothesized to occur following 13-*cis*-RA treatment. A hyposerotonergic state is thought to occur through a compensatory reduction in 5-HT turnover, reduced 5-HT synthesis and/or increased 5-HT uptake (although the evidence is only strong for increased 5-HT uptake, significant findings are denoted by *). There is also evidence of alterations in the dopaminergic pathway and may impact upon serotonergic systems. Either the hyposerotonergic state or altered dopaminergic may subsequently mediate the depression-related behaviour observed after 2 and 6wks of 13-*cis*-RA treatment in rats and mice in the resident-intruder paradigm, FST and TST. Current findings in red, and previous findings in black.

In fact, the most consistent molecular alteration observed in this thesis was increased protein levels of D2DR, which was expected given its use as a positive control and may also highlight a change in dopaminergic pathways. However, this is in contrast to the current understanding of the molecular mechanisms underlying 13-*cis*-RA-mediated depression, which is dominated by serotonergic

alterations. However, it may be possible that dopaminergic dysregulation is able to affect serotonergic pathways (or vice versa), although the mechanisms behind this will require elucidation (also see Chapter 1.3.1.5.).

Alternatively, monoaminergic pathways may play no role in 13-*cis*-RA-induced depression and changes in other brain neurotransmitter systems may be of importance such as glutamate and GABA, or perhaps through reduced levels of neurogenesis (see Chapter 1.3.1.6) (Belmaker *et al.*, 2008; Crandall *et al.*, 2004).

The findings and conclusions of this thesis that 13-*cis*-RA can induce pro-depressive could have important implications at the clinical level. Although the original manufacturers of 13-*cis*-RA, Roche, have officially discontinued the manufacture and distribution of Accutane in the USA (Accutane product deletion, 2009) due to both a low market share and from the high costs of personal-injury lawsuits (Genentech, 2009), 13-*cis*-RA is still widely prescribed for acne suffers in the form of generic versions such as Claravis, Isotane, Oratane, ISOTRET, Sotret, Isotrex and Isotrexin. Furthermore, in patients where antibiotic treatment with erythromycin or tetracycline has proven to be ineffective, there is no effective alternative treatment other than 13-*cis*-RA (Langner *et al.*, 1985; Zouboulis *et al.*, 2003). My findings are therefore relevant to all 13-*cis*-RA acne patients, the clinicians that diagnose and prescribe 13-*cis*-RA and the MHRA that assess the merits and safety of drugs in the UK. The MHRA has in fact conducted a review of incidents of depression, suicide ideation and completed suicide amongst Roaccutane patients (see Chapter 1.3.3.2.) and clearly stated the need for vigilance when prescribing Roaccutane (Isotretinoin report MHRA UK, 2004).

However, it is clear that the onset of depression following 13-*cis*-RA treatment occurs in a subset of patients and must be balanced against the efficacious ability of 13-*cis*-RA to treat acne and the positive effects this will have on the individual patient. Furthermore, results from the resident-intruder paradigm suggest that the pro-depressive phenotype induced by 13-*cis*-RA is reversible after 1 week of discontinuation. Ideally, the advent of new safer drugs entering the marketplace in the future such

as SMT D002, proposed to be highly efficacious at reducing sebum production by 90% in 18 patients during a Phase I clinical trial with no noted side-effects ((Summit plc, 2008)), will reduce the usage of 13-*cis*-RA.

More fundamentally, the findings that excessive retinoids can mediate the onset of depression adds to the new and emerging interest in Vitamin A and retinoid signalling in the adult brain that is quite distinct from the well-established studies of retinoids in the developing brain. Many aspects of the retinoid signalling pathway have been uncovered, although there remains a great deal that requires further study such as the array of neuronal (and non-neuronal) genes which are able to initiate gene transcription through retinoids and the normal physiological role of the retinoid signalling system in the adult brain. With regards to the latter point, the functional role of the retinoid signalling pathway in the adult brain is thought to include learning and memory, synaptic plasticity and locomotion, whereas dysregulation of the retinoid signalling pathway is involved in Alzheimer's disease and schizophrenia (Lane *et al.*, 2005). Therefore, the findings of my thesis suggest a new array of retinoid-sensitive neuronal genes that may provide new avenues of research and strengthens the growing consensus that retinoid signalling dysregulation is involved in neurological disorders.

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Appendix

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